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Novel Modulators of Human Sperm Motility

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Table of Contents

List of Figures and Tables 6

Acronyms and Abbreviations 8

Acknowledgements 10

Declaration 10

Summary 11

CHAPTER 1 General Introduction 12

1.1 Male infertility 13

1.2 Diagnosis of male infertility 13

1.2.1 Semen analysis 13

1.2.2 Sperm Function Assays 14

1.3 Asthenozoospermia 15

1.4 The Use of ART 15

1.5 Capacitation 16

1.6 Initiation of sperm motility 17

1.6.1 Hyperactivation 18

1.7 The role of Ca^{2+} signalling in sperm motility 21

1.7.1 Calcium channels in sperm 21

1.7.2 Calcium stores in sperm 27

1.8 Flexstation screening assay 28

1.9 Chemical stimulation of sperm motility 29

1.10 Computer assisted sperm analysis (CASA) of sperm motility 30

1.11 Thesis Aims 32

CHAPTER 2 Methods and Materials 34

2.1 Reagents and Stocks 35

2.2 Media used for donor samples 36

2.3 Samples 36

2.3.1 Ethical Approval 36

2.3.2 Semen Samples 37

2.4 Sperm preparation 38

2.5 CASA parameters adopted in all experiments 38

2.6 Gradient experiment (Chapter 3) to determine most suitable fraction to be used as a surrogate for patients 39

2.7 Assessment of calcium and motility induced by novel compounds 39

2.7.1 Flexstation Assay Screen (experiments conducted by Sarah Matins da Silva in combination with the Dundee Drug Discovery Unit (DDU)) 39

2.7.2 Preparation for motility assessment of novel compounds 40
2.7.3 Sperm penetration into artificial viscous medium (Kremer test) 41
2.8 Assessment of the Acrosome Reaction 42
2.9 Hypo-osmotic swelling (HOS) test 43
2.10 Statistical analysis 44
CHAPTER 3 Identification of a sub-group of donor spermatozoa after density gradient centrifugation that is comparable to a patient sample 45
3.1 Introduction 46
3.2 Results 48
3.3. Discussion 53
CHAPTER 4 Development of Standard Operating Procedure for Drug Screening 55
4.1 Introduction 56
4.1.1 Minimum number of cells counted to reduce sampling error 57
4.1.2 Variation in motility assessed by different slide types 57
4.1.3 Between-donor and within- donor biological variation 58
4.1.4 Effect of DMSO on sperm motility 59
4.2 Results 60
4.2.1 Development of methods for motility assessment by CASA 60
4.2.2 Development of methods for the assessment of motility for drug screening 65
4.3 Discussion 69
4.3.1 Repeatability and Reliability of system 69
CHAPTER 5 Motility Analysis of Sperm treated with Hit Compounds identified through a Flexstation High Throughput Screening Assay 76
5.1 Introduction 77
5.1.1 Cannabinoid agonists 79
5.1.2. Mitogen activated protein kinases 81
5.1.3 c-MET kinase inhibitor 83
5.2 Experimental Design 85
5.3 Results 85
5.3.1 Motility assessment by CASA of five hit compounds 85
5.4 Discussion 94
CHAPTER 6 Motility Analysis of the PDE inhibitor Trequinsin Hydrochloride 100
6.1 Introduction 101
6.1.1 Phosphodiesterase inhibitor 101
6.2 Experimental Procedure 103
6.3 Results 104

6.3.3 Vitality analysis (HOS test) of treated sperm cells 112
6.3.4 Response of patient samples to incubation with Trequinsin Hydrochloride 113
6.3 Discussion 116
CHAPTER 7 Investigating the effects of novel ion channel compounds on sperm motility 119
7.1 Introduction 120
7.2 Experimental Procedure 122
7.2 Results 122
7.2.1 Motility assessment by CASA of five hit compounds 122
7.2.2 Evaluation of sperm motility into artificial viscous medium (Kremer test) 125
7.2.3 Evaluating acrosome reaction in response to agonist stimulation 129
7.2.4 Vitality assessment of sperm cells exposed to agonists 131
7.2.5 Effect of ion channel agonists on patient samples 132
7.3 Discussion 135
CHAPTER 8 Investigating the incidence of sub-fertile patients, from andrology clinics, who could benefit clinically from sperm stimulants 138
8.1 Introduction 139
8.2 Experimental design 142
8.3 Results 143
8.4 Discussion 151
CHAPTER 9 Conclusion and Future work 153
References 159
CHAPTER 10 Appendix 177
10.1 Components, product list and catalogue numbers for Media and Compounds used. 178
10.2 Comparing donor fractions to 80% fraction recovered from patients. 179
10.3 Determination of the number of aliquots from a single sample that need to be taken to assess motility parameters 185
10.4 Determination of slide type required for CASA assessment of motility 189
10.5 Assessment of motility parameters in capacitating and non-capacitating media 190
10.6 Effect of 1% DMSO on treated sperm samples 191
10.7 Treatment with Leelamine Hydrochloride 193
10.8 Treatment with GP1a 195
10.9 Treatment with EO1428 197
10.10 Treatment with JX401 199
10.11 Treatment with PHA665752 201
10.12 Treatment with Trequinsin Hydrochloride 203
10.13 Treatment with Novel Ion Channel Compounds (A1-H1) 206

10.14 Progesterone Metabolites (Neurosteroids) 209
10.14.1 Introduction 209
10.14.2 Results 211
10.14.3 Discussion 214
10.15 Clinic A and B datasets 216
10.16 Consent form for patient/donor participation in research 220
References 222

List of Figures and Tables

Figure 1-1 Schematic of motility patterns identified during capacitation. 20
Figure 1-2 Diagram of labelled Human Spermatozoa indicating position of CatSper channel 23
Figure 1-3 Role of human KSper (Slo1) in sperm physiology and hyperactivation 26
Figure 1-4. Schematic identifying motility trajectories as determined by CASA. 31
Figure 2-1 Model depicting sperm penetration into artificial viscous medium (Kremer test) 42
Figure 2-2. Image depicting acrosome intact (AI) and acrosome reacted (AR) spermatozoa. 43
Figure 3-1Comparison between donor and sub-fertile samples in Percoll fractions relative to incubation time under capacitating conditions 49
Figure 3-2 Comparison between donor and sub-fertile samples in Percoll fractions relative to incubation time under capacitating conditions 51
Figure 4 Examples of slide types examined 57
Figure 4-1 Key for identifying features in Box Plots 60
Figure 4-2. Variation in range of results 4 aliquots vs 2 aliquots in non capacitating and capacitating buffer 61
Figure 4-3. Variation in motility between two slide types 62
Figure 4-4. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating media (CM) for 40% and 80% fractions. 64
Figure 4-5 Inter-ejaculate variation in 5 donors following two assessments 65
Figure 4-6. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating (CM) media treated with 1% DMSO for 80% fraction 68
Table 5.1 Summary of active compounds identified in screen of the Chemogenomics library examined for their effects on sperm motility 78
Figure 5-1. Key signalling components of the four major MAPK pathways in mammals 81
Figure 5-2 Proposed model for p38 and ERK1/2 signalling in human sperm. 82
Figure 5-3. Heuristic model by Naz et al showing tyrosine phosphorylation signalling pathways in human sperm cells involved in capacitation. 84
Figure 5-4. Expression of basal (control) motility values compared to Leelamine Hydrochloride treated samples capacitating conditions. 86
Figure 5-5 Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating media (CM) 87
Figure 5-6. Expression of basal (control) motility values compared to EO1428 treated samples in non-capacitating (NCM) and capacitated (CM) media. 90
Figure 5-7. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating media. 92
Figure 5-8. Expression of basal (control) motility values compared to treated samples in capacitating (CM) media in 80% fraction. 93
Figure 5-9 The endocannabinoid system and sperm function 95
Table 6.1 Summary on Trequinsin Hydrochloride identified from screen of the Chemogenomics library 101
Figure 6-1.Signaling pathways involved in sperm capacitation. 102
Figure 6-2. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating (CM) media. 106
Figure 6-3. Comparing basal (control) motility values under capacitating conditions to Trequinsin treated non-capacitated samples. 109
Figure 6-4 Trequinsin induced penetration of sperm cells into viscous medium 110
Figure 6-5. Vitality analysis after incubation with Trequinsin. 112
Table 6.2 Identification of motility kinematics analysed by CASA, from patient samples, after incubation with Trequinsin Hydrochloride. 114

Figure 6-6 Agonist induced sperm penetration into viscous medium.....114
Figure 7-1 Expression of basal (control) motility values compared to treated samples in non-capacitating media (NCM).....122
Figure 7-2. Expression of basal (control) motility values compared to treated samples in capacitating media (CM), 80% fraction for % progressive motility.. 124
Figure 7-3 Agonist induced penetration of cells into viscous medium (80% fraction).....125
Figure 7-4 Agonist induced penetration of cells into viscous medium (40% fraction).....127
Figure 7-5 Agonist induced acrosome reaction.....129
Figure 7-6. Vitality analysis after agonist exposure..... 131
Table 7.1 Identification of motility kinematics, measured by CASA, from patient samples after exposure to B1.. 133
Figure 7-7 Agonist induced sperm penetration into viscous medium.....133
Figure 8-1 Figure courtesy of Publicover et al denoting likelihood of conception with regard to the concentration of motile cells. 141
Figure 8-2 Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic A (2002-2003) 144
Figure 8-3. Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic B (2011)..... 144
Figure 8-4 Scatter plots identifying correlations for clinic A.....144
Figure 8-5 Scatter plots identifying correlations for clinic B.....145
Figure 8-6 Cumulus frequency graph depicting the percentage of sub-fertile patients suitable for treatment with motility enhancing compounds from clinic A and B 150

Acronyms and Abbreviations

ACU: Assisted conception unit

AI: Artificial insemination

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

AR: Acrosome reaction

ART: Assisted reproductive technology

BCF: Beat-cross frequency (Hz)

BSA: Bovine serum albumin

Ca^{2+}: Calcium ions

$[Ca^{2+}]_i$: Intracellular calcium ions

cADPR: Cyclic ADP ribose

CAM: Calcium/ calmodulin

CAMK II: Calcium/ calmodulin dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CASA: Computer-aided sperm analysis

Ca_v: Voltage gated calcium channels

cGMP: Cyclic guanosine monophosphate

CICR: Calcium induced calcium release

CNG: Cyclic nucleotide-gated

CM: Capacitating medium

CO_2: Carbon dioxide

DDU: Drug discovery unit

DGC: Density gradient centrifugation

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

E_m: Membrane potential

HCO_3^-: Bicarbonate ions

HFEA: Human Fertilisation and embryology association

HOS: Hypo-osmotic swelling

HTS: High throughput screening

HVA: High voltage activated

ICSI: Intracytoplasmic sperm injection

IP_3Rs: Inositol triphosphate receptors

IUI: Intrauterine insemination

IVF: In-vitro fertilisation

K^+: Potassium ions

LIN: Linearity

LVA: Low voltage activated

Na^+: Sodium ions

NAADP: Nicotinic acid–adenine dinucleotide phosphate

$NaHCO_3$: Sodium bicarbonate

NCM: Non- capacitating HEPES-buffered medium

pH_i: Intracellular pH

PKA: Protein kinase A

PKC: Protein kinase C

PTX: Pentoxifylline

ROS: Reactive oxygen species

r.p.m.: Revolutions per minute

RT-PCR: Real time polymerase chain reaction

RyRs: Ryanodine receptors

sAC: Soluble adenylate cyclase

SD: Standard deviation

SEM: Standard error of the mean

STR: Straightness (VSL/VAP)

TMC: Total motile count

tmACs: transmembrane adenylate cyclases

UoD: University of Dundee

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight-line (rectilinear) velocity

WHO: World Health Organization

WOB: Wobble (VAP/VCL)

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Declaration

I declare that the content of this project report is my own work and has not previously been submitted for any other assessment. The report is written in my own words and confirms to the University of Dundee's Policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report.

Signature: Date:

Summary

Male factor is the underlying cause in 50% cases of infertility, and is most commonly characterised by asthenozoospermia (reduced sperm motility). Incredibly, there is currently no known treatment for this condition, and the only option is Artificial Reproduction Technology (ART) which is expensive and invasive. The development of a novel treatment would fulfil a therapeutic niche that needs to be met. Using calcium as a surrogate for motility, a Flexstation high-throughput screening assay (HTS) of 3312 drug discovery ion channel compounds was performed. The screen identified 14 hit compounds that increase intracellular calcium in human spermatozoa. This project investigated these compounds, along with, 6 hit compounds previously identified from a HTS of a Chemogenomics library (246 compounds) for their potential to enhance sperm motility. One compound, Trequinsin hydrochloride (Chemogenomics library), produced statistically significant increases in progressive motility (P=0.02) instantaneously (t=0 min), an effect that was sustained for 3 hrs. Trequinsin hydrochloride, a potent class 3 phosphodiesterase inhibitor, along with B1, an ion channel compound, produced statistically significant increases in penetrating ability of spermatozoa through viscous medium. These compounds were both examined on sub-fertile patient samples, with positive results, providing preliminary data on their clinical relevance.

CHAPTER 1

General Introduction

1.1 Male infertility

Infertility is most commonly defined by the failure to conceive after a minimum of 12 months of regular unprotected intercourse (Rowe, 1993, Irvine, 1998, NICE, 2013). In the UK an estimated 3.5 million people suffer from infertility (HFEA 2011). Information for prevalence of infertility worldwide is based on data from either clinically based, (Thonneau et al., 1991, Hull et al., 1985) or population based, (Anderson et al., 2009, Walschaerts et al., 2013) studies and are in agreement that approximately one in six couples worldwide will have problems conceiving during their reproductive life (Irvine, 1998, Boivin et al., 2007). A common factor in each of these studies is that male factor infertility is the single most common diagnostic category (Hull et al., 1985, Thonneau et al., 1991, Schmidt et al., 1995), thus, male sub-fertility is a significant global problem and its prevalence is continuing to negatively impact on artificial reproductive technology (ART) (Barratt et al., 2011, Tomlinson et al., 2013). There is an urgent requirement to develop new tests for accurately diagnosing male infertility and new treatments that are specific to male factor conditions.

1.2 Diagnosis of male infertility

1.2.1 Semen analysis

It is difficult to diagnose male infertility and the universal approach that is most often adopted is using conventional criteria described by the World Health Organisation (WHO, 1987, WHO, 1992, WHO, 2010e). This criterion is solely based upon a descriptive semen analysis involving the assessment of morphology, motility and sperm concentration. However, it has generally been accepted that semen analysis is of little clinical value for diagnosing infertility (Tomlinson et al., 1999, Agarwal and Said, 2011) with the exception of circumstances where the man has very low numbers of motile sperm (Macleod and Gold, 1951a). Many groups have examined the robustness of semen analysis as a tool for diagnosing infertility with similar conclusions being

ascertained as the first study by Macleod and Gold in 1951 (Macleod and Gold, 1951a, Cooper et al., 2010). Since this conclusion that semen analysis is of limited value in the prediction of male fertility and fertilising ability, there has been a need to develop further assessments for evaluating male fertility. To fill this area methods have been developed that evaluate sperm functional competence.

1.2.2 Sperm Function Assays

A number of assays have been developed that are capable of assessing sperm function which evaluates the capabilities of sperm to progress through the female reproductive tract and interact with the oocyte (Aitken, 2006, Lefievre et al., 2007, Barratt et al., 2011). However, currently these tests have yet to make a significant impact on management of infertility in a clinical setting (Lefievre et al., 2007, Barratt et al., 2011).

Sperm motility is one of the most important sperm functions generally analysed through semen analysis (Macleod and Gold, 1951a, Curi et al., 2003, Darszon et al., 2011). Ca^{2+} plays a fundamental role in motility, specifically in hyperactivated motility (Ho, 2001, Darszon et al., 2011, Barratt et al., 2011). Current knowledge on how Ca^{2+} is regulated by the cell has increased substantially and although there are still gaps in our understanding, clinical research suggests that Ca^{2+} regulation could be used as a means of identifying sperm dysfunction (Krausz et al., 1996, Costello et al., 2009, Barratt et al., 2011). Currently there are few options available for diagnosing male infertility both clinically and in the laboratory and for this reason semen analysis was used in this study, along with functional assays, including the Kremer mucus penetration test, to determine fertilising capabilities of spermatozoa.

The Kremer sperm penetration test examines the sperm-mucus interaction *in vitro*. It makes use of a glass-capillary tube filled with cervical mucus or a cervical mucus

substitute (methylcellulose) which is placed in contact with a sperm sample (Kremer, 1965, Ivic, 2002). The test predominantly highlights the proportion of progressively motile sperm in a sample identifying the number of sperm capable of mucus penetration (Katz et al., 1980). This is a simple and effective *in vitro* test known to provide important information about sperm function (Barratt et al., 1989, Abu-Heija et al., 1996, Ivic, 2002).

1.3 Asthenozoospermia

Asthenozoospermia (low sperm motility), is defined clinically by a semen analysis where the total number of motile cells is less than 40% and/or the percentage of progressively motile cells is less than 32% (WHO, 2010b). A large retrospective study determined that 81% of infertile men are diagnosed with asthenozoospermia, 19% of these men had isolated asthenozoospermia and 63% had asthenozoospermia in combination with teratozoospermia (poor sperm morphology) and/or oligozoospermia (low sperm concentration) (Curi et al., 2003). A low sperm motility can be caused by structural (Chemes et al., 1998, Imai et al., 2001, Visser et al., 2011) or functional deficiencies (Makler et al., 1980), by a deleterious effect of seminal plasma (Mortimer et al., 1998) or by a combination of these factors. There is currently no drug a man can take, nor can be added to sperm *in vitro* to increase sperm motility (Barratt et al., 2011).

1.4 The Use of ART

Currently the main treatment options available for motility dysfunction or sperm dysfunction, as a whole, is ART. ART comprises of a range of treatments according to the severity of sperm dysfunction, i.e. for mild male factor sub-fertility the treatment is IUI (Intra Uterine Insemination), for moderate IVF (In Vitro Fertilisation) and for severe ICSI (Intra Cytoplasmic Sperm Injection), all of which are expensive and invasive (Tournaye, 2012). The use of ART worldwide is increasing with an estimated 5

million babies being born worldwide using artificial reproductive technology (ART) (ESHRE, 2012). However, the cost of ART is a major deterrent to couples who need treatment in both developing and developed countries (Rauprich et al., 2010). Sperm movement characteristics and sperm morphology are significantly, positively correlated with fertilisation rates *in vitro* (Sukcharoen et al., 1995, Sukcharoen et al., 1996) suggesting that the ability to develop compounds that could increase sperm movement characteristics would have a positive effect on *in vitro* fertilisation rates.

1.5 Capacitation

Capacitation (Austin, 1951, Chang, 1951) is the term given to the processes that sperm need to undertake *in vivo* in the female reproductive tract to acquire the ability to fertilise oocytes. Sperm capacitation was first discovered independently by Austin and Chang in rats and rabbits (Austin, 1951, Chang, 1951). The process of capacitation is poorly defined owing to there being no clear marker to indicate the occurrence of capacitation (Breitbart, 2002). However, capacitation has been characterized as a complex of changes (structural and functional) that occur in the spermatozoon after a period of time spend in the female reproductive tract (De Jonge, 2005). Some of these changes in mammals include: increases in intracellular Ca^{2+} and cAMP concentrations along with changes in the lipid composition of the plasma membranes resulting in a net cholesterol decrease (McLeskey et al., 1998, O'Flaherty et al., 2006, de Lamirande and O'Flaherty, 2008, Buzadzic et al., 2014). The removal of cholesterol and other sterols (de-capacitating factors) from the sperm plasma membrane, during capacitation, along with the non-covalently attached glycoproteins being released from the epididymis, produces a more fluid membrane environment that prime the spermatozoa for further fertilisation cues (Ikawa et al., 2010). During capacitation changes also occur in oxidative metabolism, tyrosine phosphorylation, sperm motility (altering to the hyperactivated state) and a drastic reorganisation, removal or modification of proteins

within the spermatozoa. Changes in the sperm plasma membrane are essential for the initiation of sperm motility, hyperactivation and induction of the acrosome reaction (Gadella and Visconti, 2006). The ability of the spermatozoa to hyperactivate and undergo the acrosome reaction are essential for fertilisation (Bavister, 2002, Liu et al., 2007). If spermatozoa are unable to capacitate they will be unable to fertilise an oocyte successfully without assistance (Fraser, 1998).

1.6 Initiation of sperm motility

Mammalian spermatozoa are not motile in the male body they acquire motility after ejaculation (Lindemann and Kanous, 1989, Si and Okuno, 1995, Buzadzic et al., 2014). Motility is usually initiated by changes in environmental conditions, such as ionic concentrations or osmotic stimulation (Morisawa, 1994). During capacitation, early events which are regulated by a HCO_3^-/ soluble adenylyl cyclase (sAC) /cAMP/ protein kinase A (PKA) pathways initiate sperm motility (Lee and Storey, 1986, Boatman and Robbins, 1991b, Salicioni et al., 2007, Visconti, 2009). Sperm motility is initiated immediately after sperm are released from the epididymis after contact with high concentrations of HCO_3^- in the seminal plasma and female reproductive tract ($\geq$20mM) (Chen et al., 2000, Zhou et al., 2005, Xie et al., 2006). HCO_3^- plays an important role in mediating sperm motility and its actions are determined by activation of sAC, which initiate an increase in cytoplasmic cAMP levels (Chen et al., 2000, Xie et al., 2006, Battistone et al., 2013). This elevated cAMP level triggers activation of PKA which stimulates serine/threonine phosphorylation of target proteins, and ultimately, activates tyrosine phosphorylation of proteins located mainly in the flagellum in mammals (Carrera et al., 1996, Naz and Rajesh, 2004, Battistone et al., 2013). Knock out studies have been conducted on sAC and PKA null mice resulting in infertile males, however, addition of cell permeant cAMP reinstates flagella beat and sperm motility (Esposito et al., 2004, Xie et al., 2013). This indicates that the motility impairment noted is not due

to a structural alteration or defect in the sAC null mice (Esposito et al., 2004, Xie et al., 2013).

As previously described, during capacitation, cAMP levels increase resulting in tyrosine phosphorylation, which, is one mechanism for maintaining sperm motility. In addition to this the membrane potential of the cell becomes hyperpolarised and intracellular pH (pH_i) along with intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in both mice (Visconti et al., 1995b), and humans (Brewis et al., 2000, Visconti et al., 2002). Mature sperm stored in the epididymis are quiescent due to the male reproductive tract having an acidic pH (~5.5 to 6.5). Sperm motility is initiated on ejaculation when sperm are mixed with seminal plasma (extracellular pH>7) which cause the pH_i to increase within the cell to approximately 6.8 (Hamamah et al., 1996), subsequently, while traversing the female reproductive tract, the pH_i is elevated further (Hamamah et al., 1996, Lishko et al., 2012b, Kirichok and Lishko, 2011). Intracellular alkalinisation is another important factor that regulates the initiation of sperm motility, capacitation and hyperactivation (Hamamah et al., 1996, Suarez, 2008, Lishko and Kirichok, 2010).

1.6.1 Hyperactivation

Hyperactivation is the term given to the change in sperm swimming and is a concomitant event with capacitation in humans, it involves a change from symmetrical flagella beats to high amplitude, asymmetrical flagella bending (Suarez, 2008). It was first described in sperm from hamsters undergoing capacitation *in vitro* and similar patterns of movement were observed *in situ* through the walls of the oviductal ampulla of golden hamsters at the time of fertilisation suggesting a potential role in fertilisation (Yanagimachi, 1970). Hyperactivation has since been observed *in vitro* in spermatozoa from a variety of mammals including; rabbit (Johnson et al., 1981), mouse (Fraser,

1977), dog (Mahi and Yanagimachi, 1978), bull (Singh et al., 1983) and human (Mortimer et al., 1984). Hyperactivated motility is a property of the flagella although it is often measured according to changes in head movement. The changes observed between non-hyperactivated and hyperactivated spermatozoa are results of changes in the degree of axonemal bending along with changes in the propagation of flagella beats (Mortimer et al., 1997). Studies on human sperm capacitation have identified three main patterns of movement: (i) forward progressive motility (figure 1-1A), (ii) transition motility which is similar to progressive motility but has a more irregular track (figure 1-1B) and (iii) hyperactivated motility (figure 1-1C) (Mortimer and Mortimer, 1990). *In vitro*, hyperactivated spermatozoa have been described as swimming in 'circles' or having a 'starspin' motion (Ho and Suarez, 2001, Mortimer and Swan, 1995, Burkman, 1991). The hyperactivated movement pattern of human sperm is 'biphasic', similarly to rabbit sperm, meaning that the spermatozoa switch between a whiplash (hyperactivated) phase and a progressive phase (non-hyperactivated), depending on the environmental cues from the female reproductive tract and oocyte vestments, allowing the cell to alter its motility according to the requirements of the environment (Pacey et al., 1995, Mortimer and Swan, 1995, Mortimer and Mortimer, 1990, Olson et al., 2011).

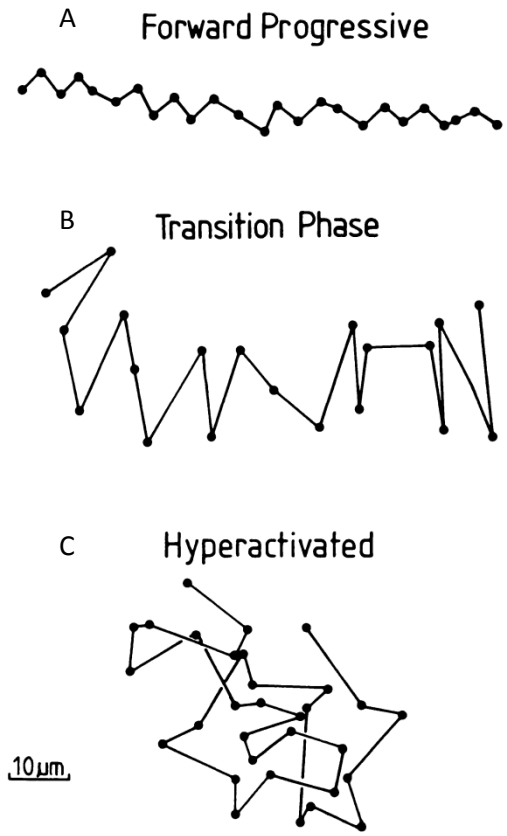


Figure 1-1 Schematic of motility patterns identified during capacitation. (A) forward progressive motility, **(B)** transition phase – moving in a forward direction but with larger 'whip-like' action and **(C)** hyperactivated motility. Capacitated sperm are biphasic and switch between the three patterns of movement. **(Mortimer and Mortimer, 1990)**

Hyperactivation is crucial to sperm function aiding the sperm to travel along the female reproductive tract. The exact role that hyperactivation plays is still debated but, evidence suggests, it may facilitate migration through the highly viscous cervical mucus and enable penetration of the layers surrounding the oocyte (Suarez et al., 1991, Suarez, 2008, Ren et al., 2001). Experiments have also shown that hyperactivation may be required to detach the sperm from the oviductal epithelium in both animals and humans (Demott and Suarez, 1992, Pacey et al., 1995). Clinical studies in humans have provided data identifying that the percentage of hyperactivated sperm cells correlates with successful fertilisation *in vitro* (Sukcharoen et al., 1995). The central regulator of hyperactivated motility is an elevated $[Ca^{2+}]_i$ level which is required for both initiation and maintaining the hyperactivated state (Alasmari et al., 2013a). There are at least two sources of Ca^{2+} that regulate the hyperactivated state in mammalian sperm these are;

entry through pH dependent CatSper channels located in the plasma membrane of the principle piece of the flagella (Ren et al., 2001, Olson et al., 2010, Ren and Xia, 2010) and mobilisation of stored Ca^{2+} from the neck/midpiece region of the sperm cell (Costello et al., 2009, Ho and Suarez, 2001).

1.7 The role of Ca^{2+} signalling in sperm motility

Sperm lack endoplasmic reticulum meaning functional regulation by transcription/ translation will be very limited. Therefore, post-translational mechanisms primarily control all activities of the cell (Costello et al., 2009). One such mechanism involves changes in intracellular Ca^{2+} concentration. $[Ca^{2+}]_i$ signalling is achieved through Ca^{2+} entering the cytoplasm (low Ca^{2+} concentration) from the extracellular space or intracellular organelles (Ca^{2+} concentration four orders of magnitude higher) (Costello et al., 2009). It is well established that Ca^{2+} permeable channels exist in the plasma membrane of sperm cells. To initiate signalling Ca^{2+} channels need to open to allow the flow of ions down their electrochemical gradient (Costello et al., 2009). Regulation of protein function through Ca^{2+} signalling plays a central role in controlling activities that are vital for sperm function, including motility, hyperactivation, chemotaxis and the acrosome reaction (Publicover et al., 2007, Darszon et al., 2011).

1.7.1 Calcium channels in sperm

1.7.1.1 *Voltage gated Ca^{2+} channels*

Voltage-gated Ca^{2+} channels (Ca_V) produce increases in $[Ca^{2+}]_i$ in response to membrane potential (E_m) changes (Darszon et al., 2011). The channels have been divided into two separate groups: high voltage activated (HVA), defined as N-type, P/Q-type, and R-type Ca^{2+} currents which require strong depolarisation for activation (Tsien et al., 1991, Catterall and Few, 2008) and low voltage activated (LVA) which were initially classified as T-type because they open transiently, need weaker

depolarisations to open and inactivate faster at more negative potentials (Darszon et al., 2011).

G-proteins have been found to regulate Ca_V channels through many different pathways, this can be via direct activation with physical interactions between the channel and the G-protein or by indirect activation via second messengers and/or protein kinases (Dascal, 2001). Ca_V channels can be stimulated by several protein kinases including PKA, protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (Welsby et al., 2003, Catterall, 2000, Catterall and Few, 2008, Dai et al., 2009). Ca^{2+}, itself, is known to inactivate Ca_V channels by associating with CaM. The mechanisms are best known for Ca_V1 and Ca_V2 channels, however it is possible that Ca_V3 channel functions could also be influenced by CaM (Lopez-Gonzalez et al., 2001). RT-PCR experiments have revealed the presence of transcripts for Ca_V1, 2 and 3 in mouse spermatogenic cells and mature human sperm (Goodwin et al., 2000, Park et al., 2003, Serrano et al., 1999). Patch clamp studies have not been able to reveal Ca_V1 or Ca_V2 channels in spermatogenic cells however; Ca_V3 currents have been well documented in spermatogenic cells in mice and humans using this technique (Darszon et al., 2006, Escoffier et al., 2007, Liévano et al., 1996). These studies suggest that these channels could be present in an electrophysiologically inactive state and may become activated during sperm maturation (Darszon et al., 2011).

1.7.1.2 *Voltage-gated proton channel H_V1*

In mammals the regulation of pH_i is fundamental for the initiation of sperm motility, capacitation, hyperactivation and the acrosome reaction (Yanagimachi, 1994). The proton selective, voltage-gated ion channel (H_V1) was cloned in 2006 and it has been reported that voltage-gated proton channels are functionally expressed in mammalian sperm (Lishko et al., 2010). H_V1 is abundantly expressed in human sperm cells and is located to the principal piece of the flagellum. This makes it perfectly located to activate

pH-dependent proteins of the axoneme and control motility (Ren et al., 2001, Kirichok and Lishko, 2011, Lishko and Kirichok, 2010). A role for H_V1 in the regulation of $[Ca^{2+}]_i$ homeostasis has been proposed involving active proton extrusion to maintain pH_i balance after CatSper activation (Lishko et al., 2012b). Potentially, a depolarizing stimulus could also create an increase in pH_i via the H_V1 channel resulting in activation of CatSper and hyperactivated motility (figure 1-2) (Ren and Xia, 2010, Mannowetz et al., 2013).

1.7.1.3 *CatSper*

CatSper is a sperm specific cation channel that facilitates the entry of Ca^{2+} into spermatozoa (Barratt and Publicover, 2012, Lishko et al., 2011). The CatSper channel is located in the principle piece of the flagellum and through this position it is able to have a key role in sperm motility (figure 1-2) (Barratt and Publicover, 2012, Ren et al., 2001).

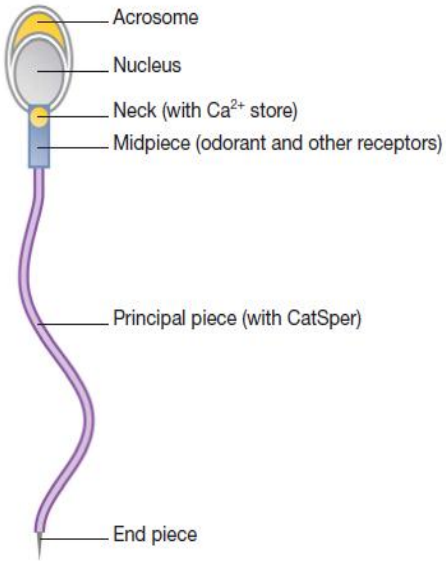


Figure 1-2 Diagram of labelled Human Spermatozoa indicating position of CatSper channel. Yellow areas highlight stores of Ca^{2+} **(Barratt and Publicover, 2012)**

The CatSper ion channel is comprised of four homologous pore-forming α subunits (CatSper 1-4) and three auxiliary subunits, CatSper β, γ and δ (Brenker et al., 2012). The Channel was identified through the use of the Patch clamp technique. Research using this technique discovered that Ca^{2+} entry into sperm triggered by progesterone is

through CatSper (Strunker et al., 2011, Lishko et al., 2011, Barratt and Publicover, 2012). An influx of Ca^{2+} is crucial for spermatozoa to be capable of fertilisation (Publicover et al., 2007) having been shown to play a role in hyperactivation, chemotaxis and the acrosome reaction (Publicover et al., 2007, Costello et al., 2009).

CatSper initiated increases in $[Ca^{2+}]_i$ can be induced by some of the most important stimuli in sperm function including cyclic nucleotides, alkaline depolarisation, zona pellucida glycoproteins and bovine serum albumin, although many of the mechanisms of action are still not understood (Ren and Xia, 2010). The CatSper channel is pH sensitive and physiologically progesterone and prostaglandin have been identified as its most potent activators (Strunker et al., 2011, Brenker et al., 2012, Barratt, 2011). Progesterone has been shown to have an effect on CatSper *in vitro* at equivalent concentrations to the normal concentrations that the sperm would encounter *in vivo* (3-5µM) (reviewed in (Baldi et al., 1998)). Both high and low concentrations of progesterone have been shown to rapidly increase $[Ca^{2+}]_i$ through Ca^{2+} induced Ca^{2+} release (CICR) (Sagare-Patil et al., 2012). Activation by progesterone causes an influx in Ca^{2+} through the CatSper channel which triggers multiple Ca^{2+} dependent physiological responses that are essential for fertilisation (Lishko et al., 2011). It had previously been believed that progesterone acted to elevate cAMP levels, which in turn, elevated $[Ca^{2+}]_i$ in human spermatozoa (Ren et al., 2001). However, Strunker *et al* were unable to confirm this suggesting that signalling in human spermatozoa is not through G-protein coupled receptor operated cAMP (Strunker et al., 2011). Strunker *et al* reasoned that the principle voltage gated Ca^{2+} channel in human sperm is CatSper.

Along with progesterone, voltage- sensitive Ca^{2+} selective currents have been shown to induce hyperactivation via CatSper. These currents are potentiated by a rise in intracellular pH suggesting that the alkalinisation that occurs during capacitation acts to

increase CatSper conductance shifting it to a more negative membrane potential (figure 1-3). This shift increases CatSper mediated Ca^{2+} entry, increasing $[Ca^{2+}]_i$, which in turn activates hyperactivation (Qi et al., 2007).

Knock out studies on mice identified that disruption to any of the four genes results in sperm abnormalities including decreased total motility, progressive motility and an inability to undergo hyperactivation rendering them infertile (Qi et al., 2007, Avidan et al., 2003). Currently in humans defects have been found on CatSper1, 2, 3 and 4 that have been linked to infertility (Visser et al., 2011). Failed fertilisations as a result of mutations were attributed to the sperm being unable to evoke a depolarisation, which is caused by Ca^{2+} entry, eliminating their ability to become hyperactivated (Hildebrand et al., 2010).

The CatSper associated progesterone receptor is sperm specific and structurally differs from the genomic form. This enables it to be a specific target for development of drugs to aid fertility or as a novel male non-hormonal contraceptive (Lishko et al., 2011, Carlson et al., 2009). Functional CatSper is only found in mature spermatozoa indicating that there should be no adverse effects anywhere else in the body if targeted specifically. HC-056456 (blocks alkaline KCl-evoked increases in $[Ca^{2+}]_i$ and $[Na^{+}]_i$) has been shown to be effective at blocking CatSper which resulted in treated sperm being unable to hyperactivate. Furthermore, addition of this compound to already hyperactivated sperm resulted in a complete reversal from the hyperactivated state back to a normal swimming pattern (Carlson et al., 2009). This indicates the importance of CatSper and Ca^{2+} in sperm motility.

1.7.1.4 *KSper*

During capacitation sperm cells undergo intracellular alkalinisation, increases in $[Ca^{2+}]_i$ and membrane hyperpolarization (Visconti et al., 2002). These processes are regulated

by sperm ion channels including H_v1 (major H^+ channel) and CatSper (major Ca^{2+} channel) in human sperm (Ren et al., 2001, Kirichok et al., 2006, Lishko and Kirichok, 2010, Lishko et al., 2011). However, until recently the identity of the main K^+ channel in human sperm remained unknown. K^+ channels are vital functional components of sperm physiology regulating membrane potential and cell motility (Mannowetz et al., 2013, Mansell et al., 2014). In mice an alkalinisation-sensitive, Ca^{2+} insensitive sperm K^+ channel (Slo3) was found to be essential for male fertility and it had been assumed that the principle K^+ channel in human sperm would have a similar molecular identity (Schreiber et al., 1998, Santi et al., 2010). However, recent examination through the patch-clamp technique in human ejaculated spermatozoa found that human K^+ currents are insensitive to intracellular alkalinisation but dependent upon $[Ca^{2+}]_i$ (Mannowetz et al., 2013). This suggested that the identity of human KSper (hKSper) may be distinct from that of murine and mice KSper which are represented by the Slo3 protein. The Slo1 channel contains structural additions allowing these channels to sense changes in voltage and $[Ca^{2+}]_i$ (Schreiber et al., 1999). Staining with anti-Slo1 antibodies selectively stained the principle piece of the flagellum which is the same location as CatSper and H_V1 (Mannowetz et al., 2013, Kirichok and Lishko, 2011). A proposed model for KSper channels indicates that KSper may act to coordinate the actions of H_V1 and CatSper during capacitation resulting in elevated levels of $[Ca^{2+}]_i$ and initiation of hyperactivated motility (figure 1-3) (Mannowetz et al., 2013).

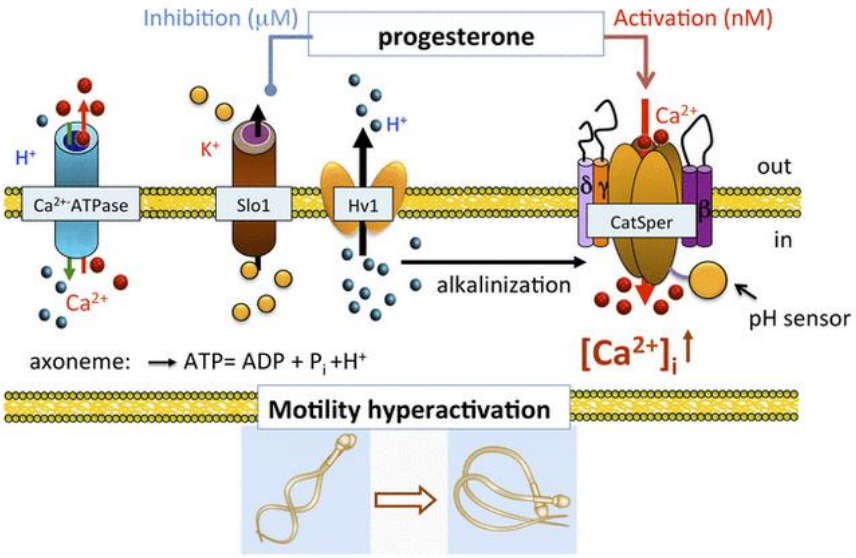


Figure 1-3 Role of human KSper (Slo1) in sperm physiology and hyperactivation.
As sperm travel through the uterus and fallopian tube they are exposed to progesterone (P) (pM – nM range), intracellular alkalinisation is evoked by Hv1 which partially activates CatSper. However, to fully activate CatSper the membrane must become depolarized. This is not achieved until sperm are in the proximity of the oocyte exposing them to micromolar concentrations of P which blocks hKSper resulting in membrane depolarization. This allows CatSper to become fully activated, triggering sperm hyperactivation and making fertilisation possible (Mannowetz et al., 2013).

1.7.2 Calcium stores in sperm

During the final stages of sperm differentiation, during nuclear volume reduction, excess material is packaged into the redundant nuclear envelope (RNE) of which an established function is unknown. This structure has been identified in sea urchin and mammalian spermatozoa and it has now been proposed that it functions as a Ca^{2+} store (Garnier-Lhomme et al., 2009, Darszon et al., 2011). For an organelle to be classified as having Ca^{2+} stores it must have two types of Ca^{2+} transporters; one enabling loading of the store and one enabling Ca^{2+} release from the store on demand (Costello et al., 2009).

Inositol trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) have been identified as the major intracellular Ca^{2+} channels located in neck/midpiece region of sperm (figure 1-2). The activity of these channels is dependent on the action of second messenger pathways such as; inositol triphosphate (IP_3), cyclic ADP ribose (cADPR)

and nicotinic acid–adenine dinucleotide phosphate (NAADP) (Zhu et al., 2010a, Zhu et al., 2010b) and they are believed to play a role in sperm hyperactivation.

1.8 Flexstation screening assay

The induction of high- throughput screening (HTS) in the 80's for drug discovery became a significant tool for identifying hits in pharmaceutical research (Terstappen et al., 2010). HTS is a well established process for lead discovery that is now being used for basic and applied research (Mayr and Bojanic, 2009). The process involves the use of automation, miniaturization assays and large scale data analysis to screen chemical libraries against a variety of extracellular and intracellular molecular targets in an attempt to identify novel chemotypes with a desired mode of action (Mayr and Bojanic, 2009). Recently the availability of large compound sources and identification of novel biological targets has dramatically changed the drug discovery process. HTS has evolved into a mature discipline that acts as a crucial source of chemical starting points for drug discovery (Mayr and Bojanic, 2009).

Complex systems such as spermatozoa that rely on post-translational modifications make discovery of new drugs for fertility fundamentally challenging. In an attempt to meet the unmet need for development of alternate treatment to ART, for male factor infertility, HTS offers a novel approach to drug discovery. HTS technology has evolved to allow for screening of focused libraries towards specific biological targets (Mayr and Bojanic, 2009, Miller, 2006). A screening library at the University of Dundee (UoD) Drug Discovery Unit (DDU) is currently being utilised in projects to identify new chemical starting points focused on ion channel targets for male factor infertility (Mok and Brenk, 2011). The passage of ions across a cell membrane is tightly regulated by ion channels. Ion channels are integral membrane proteins that are encoded by approximately 400 ion channel genes in the human genome (Mok and Brenk, 2011).

This family of membrane proteins is implicated in many important physiological functions in excitable and non-excitable cells and they underlie a wide range of diseases (Terstappen et al., 2010, Mok and Brenk, 2011, Lu and An, 2008). As a result, they are the third largest class of targets, after G-protein coupled receptors and protein kinases, in drug discovery (Lu and An, 2008, Mok and Brenk, 2011).

Martins da Silva *et al* (2012) developed and optimised a novel high throughput screening assay that assessed Ca^{2+} responses evoked by progesterone in human sperm (Martins da Silva et al., 2012). This HTS assay utilised a Flexstation microplate reader to measure increases in $[Ca^{2+}]_i$ as a response to progesterone exposure (Martins da Silva et al., 2012). This has enabled screening of a chemogenomics library, a novel UoD DDU ion channel library and a library targeting GABA A receptors to identify compounds that increase $[Ca^{2+}]_i$ in human sperm cells. The Flexstation assay uses Ca^{2+} as a surrogate for motility and is being used as the primary assay for identifying novel drugs that have the potential of modulating sperm motility.

1.9 Chemical stimulation of sperm motility

Previous attempts at improving ART fertilisation rates through chemical stimulation highlighted non-specific inhibitors of phosphodiesterases (PDEIs) e.g. pentoxifylline (PTX) as a potential treatment. PDEs hydrolyze the 3', 5'-phosphodiester bonds of cAMP or cGMP to regulate the effects of these important messengers (Lefievre et al., 2000). In 1990 Yovich *et al* (1990) released a study that identified significantly improved fertilisation rates after using PTX (Yovich et al., 1990). The use of PTX in sperm preparations, for couples undergoing fertility treatment, has also reduced the rates of failed fertilisation when dealing with male factor infertility (Yovich, 1993). PTX increased hyperactivated motility in human spermatozoa when under conditions that support capacitation (Kay et al., 1993). A further study reported higher pregnancy rates

after PTX treatment used in combination with IUI (Stone et al., 1999). However, results from PTX use were inconsistent with several groups identifying lower fertilisation rates *in vitro* along with the occurrence of a premature acrosome reaction (Tesarik et al., 1992, Ford et al., 1994, Yovich, 1993). PTX introduced the possibility of chemical stimulation for clinical application, although its use was discontinued, the potential for specific PDEi's in treating male factor infertility warrants further study.

1.10 Computer assisted sperm analysis (CASA) of sperm motility

Dedicated computer assisted sperm analysis systems have been available commercially for 25 years (Mortimer and Mortimer, 2013a) and are capable of measuring sperm motility and sperm movement characteristics (kinematics) (Mortimer, 1997). Technical issues concerning their ability to accurately determine sperm concentration in semen (Mortimer et al., 1995, Fraser et al., 1997) has discredited their use since the beginning. However, in washed preparations CASA is capable of reliably and accurately determining sperm kinematics, percentage motility and concentration provided that recommended criteria are followed (Fraser and Group, 1998). CASA analysis has proved to be invaluable in research and in infertility diagnosis (Mortimer, 2000b). It has to be noted that averaged values of sperm kinematic measures from a population alone are meaningless and to identify sub-populations that are biologically accurate measures have to be determined on a cell-cell basis by combining several kinematic parameters (Fraser and Group, 1998, Mortimer and Mortimer, 2013a). The kinematic values determined by CASA for each individual sperm cell includes the velocity of movement, the width of the sperm head's trajectory and the frequency of the change in direction of the sperm head (David et al., 1981). The velocity parameters determined are the average path velocity (VAP), which is the average direction of movement measured for the distance travelled by the sperm cell in the observable area, curvilinear velocity (VCL) which refers to the total distance covered by the sperm head and straight-line velocity

(VSL) which is determined from the straight-line distance between the first and last points of the trajectory (figure 1-4).

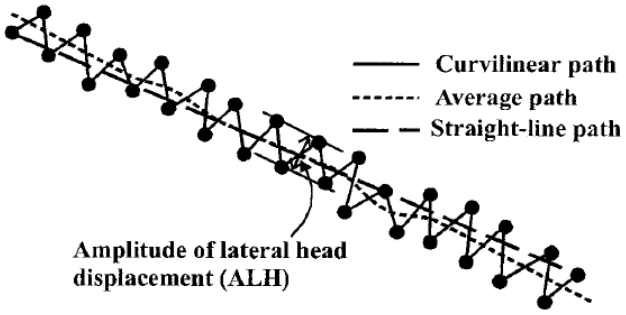


Figure 1-4. Schematic identifying motility trajectories as determined by CASA. (Mortimer, 2000b)

The shape of the trajectory influences the velocity values and three further velocities; Linearity (LIN), Straightness (STR) and Wobble (WOB) can be calculated from the ratios of the velocity values (Mortimer, 2000b). These values are determined as follows:

$$LIN = (VSL/VCL) \times 100$$

$$STR = (VSL/VAP) \times 100$$

$$WOB = (VAP/VCL) \times 100$$

The CASA also determines the amplitude of lateral head displacement (ALH) from the VAP and is calculated as the total width of the lateral movement of the sperm head (figure 1-4). Beat cross frequency (BCF) is calculated by counting the number of times the curvilinear path crosses the average path per second. This value is useful when estimating the change in the flagella pattern but a limitation of this is the limited number of observations that can be made per second, if there are more beats per second than images captured per second then the BCF will be underestimated (Mortimer and Swan, 1999, Mortimer, 2000b). To be able to determine useful information from sperm kinematic analysis a range of normal kinematic values for movement need to be identified and matched to a proportion of sperm cells in a population that meet the

criteria (Mortimer, 2000b). Using this criteria and CASA it is then possible to determine the likelihood that a proportion of spermatozoa from a semen sample are capable of penetrating his partner's cervical mucus quickly and without having to obtain cervical mucus (Mortimer, 2000b). Motility parameters such as hyperactivated motility which, under normal circumstances, is only exhibited under capacitating conditions in human sperm, analysed at 60Hz, have to meet the criteria of VCL ≥150 μm/s and LIN ≤50% and ALH ≥7.0 μm (Mortimer et al., 1998). Non-capacitated human sperm cells or sperm cells in semen normally portray parameters of motility that are defined by CASA under slow (VAP ≤ 5 μm/s), medium (VAP between 5-25 μm/s), rapid (VAP ≥25 μm/s) and/ or progressively motile (VAP ≥25 μm/s, STR ≥80%, ALH ≥ 2.5 μm and <7.0 μm) (Mortimer and Mortimer, 2013a). The benefits of CASA analysis, both in research and in the clinical laboratory, is diverse and through correct practice is able to give greater understanding of the physiological implications of sperm motility. This makes it possible to quickly predict the ability of a man's sperm to penetrate his partner's cervical mucus and to identify sperm showing hyperactivated motility under capacitating conditions to help identify the best course of treatment (Mortimer, 2000b, Mortimer and Mortimer, 2013a).

1.11 Thesis Aims

Previous research has identified the importance of Ca^{2+} signalling for sperm motility. Clinical studies have suggested a relationship between low intracellular Ca^{2+} and male factor infertility and numerous studies have highlighted the importance of sperm motility when correlated to fertilisation success. There are still many questions regarding Ca^{2+} signalling and regulation of motility in human sperm especially regarding the central role that Ca^{2+} signalling plays in sperm function. Ca^{2+} channels have been identified as potentially important targets for pharmacological interventions,

therefore, a series of experiments were designed and conducted with the following aims in mind:

1. To identify a sub-group from donor spermatozoa that can be used as a surrogate for patient samples.
2. To develop a standard operating procedure for screening compounds identified from the Flexstation high throughput screen to identify compounds that enhance motility.
3. To examine the effects of novel compounds mediating sperm motility on research sperm donors and sub-fertile patients.
4. To determine, by assessing data from two established clinics the incidence of asthenozoospermia in isolation and combination with other sperm parameters to identify patients who would benefit from treatment from therapeutic advancements and relate this to fertilisation success.

CHAPTER 2

Methods and Materials

2.1 Reagents and Stocks

Stock solutions of 1% dimethyl sulfoxide (DMSO) in distilled water were prepared on a monthly basis and stored at room temperature until use.

To make the stocks for PHA665752, inhibitor of MET kinase and Leelamine hydrochloride (CB_1 agonist), each were dissolved in DMSO to a concentration of 80mM and stored in the freezer (-20^{o}C) (source and catalogue numbers see appendix). These 80mM stocks were further diluted in dH_2O to make aliquots with a final concentration of 4mM. The final concentration of PHA665752 and Leelamine hydrochloride when in the sperm suspension was 40µM, which is the same concentration the compounds were screened at through the Flexstation assay screen, with final concentration of DMSO not exceeding 1%.

Stock solutions for GP1a (CB_2 agonist), JX401 (inhibitor of p38α), EO1428 (inhibitor of p38α and p38β) and Trequinsin hydrochloride (inhibitor of cGMP-inhibited phosphodiesterase, PDE3) were prepared by dissolving in DMSO (GP1a, EO1428 and Trequinsin hydrochloride 80mM, JX401 70mM), and stored in the freezer (-20^{o}C) (source and catalogue numbers see appendix). Aliquots of these stocks were further diluted in DMSO to a final volume of 4mM, and stored in the freezer (-20^{o}C) for no longer than 4 months. The final concentration of each; GP1a, JX401, EO1428 and Trequinsin hydrochloride, in sperm suspension was 40µM to ensure it matched the concentration of the compounds in the Flexstation assay screen, with a final concentration of DMSO not exceeding 1%.

Stock solutions for A1-H1 and A2-G2 were produced by the UoD DDU at a concentration of 10mM made up in 100% DMSO and further diluted in dH_2O to a concentration of 1mM and stored at 4^{o}C. The final concentration of each of these

compounds in sperm suspension was 10μM (same concentration as used in the Flexstation screen) with a final concentration of DMSO not exceeding 1%.

2.2 Media used for donor samples

A non- capacitating HEPES-buffered medium (NCM) was used to measure samples under a non-capacitated state, consisting of: 1.8mM $CaCl_2$, 5.4mM KCl, 0.8mM $MgSO_4.7H_2O$, 116.4mM NaCl, 1.0mM $NaH_2PO_4.2H_2O$, 5.55mM D-glucose, 2.73mM sodium pyruvate ($C_3H_3NaO_3$), 41.75mM sodium lactate ($NaC_3H_5O_3$), 25mM HEPES, 0.3% BSA dissolved in 10mls H_2O (total volume: 50ml) (Matson and Tardif, 2012, Alasmari et al., 2013a). The pH was adjusted to 7.4 by the addition of 1M NaOH with an osmolarity of 290-320mOsm/kg. A synthetic tubal fluid (STF) was used as a capacitating medium (CM) (adapted from NCM). This was used to measure samples under capacitating conditions derived similarly to NCM but containing 26mM sodium bicarbonate ($NaHCO_3$) instead of HEPES and 25mM $NaC_3H_5O_3$ instead of 41.75mM (in NCM). The STF was adjusted to pH 7.4-7.6 by the addition of 1M NaOH with an osmolarity of 290-320mOsm/kg (source and catalogue numbers see appendix).

2.3 Samples

2.3.1 Ethical Approval

Volunteer sperm donors (healthy men selected at random from the general public (mainly students) with no known fertility problems) were recruited in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) (covers donor recruitment) under local ethical approval (08/S1402/6). Written consent was obtained from patients in accordance with the HFEA Code of Practice (version 8) under the same ethical approval as stated above from the Tayside Committee of Medical Research Ethics B (patient and donor participation, see appendix for consent forms).

2.3.2 Semen Samples

Volunteer donor semen samples were produced by masturbation into a sterile plastic container following 2-3 days sexual abstinence. Samples were produced in the morning and brought to the lab within 1 hour (hr) of production. The samples were left to liquefy at 37^{o}C for approximately 30 min before being analysed.

Semen samples collected from patients were produced in the Assisted Conception Unit (ACU) by masturbation into a sterile container, following 2-3 days abstinence, on the morning of treatment, and prepared by the ACU laboratory by density gradient centrifugation. 1.5ml gradients were layered using 40% and 80% PureSperm® with 2ml semen overlaid on top. After centrifuging at 300*g* for 20 min the 80% fraction was recovered and washed in 5ml SAGE® gamete buffer which was centrifuged at 500*g* for 10 min. If the sample was used in an ICSI procedure the required amount of 80% fraction was left in 100µl SAGE® gamete buffer. If used for IVF the 80% fraction was re-suspended in Quinn's Advantage® fertilisation medium supplemented with human serum albumin (HSA). Surplus sample not required for treatment were used in research. 'Morning' samples were prepared in the ACU laboratory by PureSperm® DGC (described above) (source and catalogue numbers see appendix). A proportion of the 80% fraction is removed to be used for treatment whilst the remainder of the 80% and the 40% fraction are combined back together and made available for research. The sample is then re-prepared in the research laboratory (to re-separate sperm into 40% and 80% fractions), by Percoll® density gradient (described below), and the 80% fraction collected. Samples used in chapter 3 represent patient samples donated to research following ACU embryologist preparation that were then re-prepared in the research laboratory. 'Afternoon' samples were prepared by embryologist preparation in the ACU (same as morning samples described above) however; these prepared samples were capacitated for a minimum of 3 hrs because they had been placed in the CO_2 incubator 2

hrs prior to being used for insemination (IVF) in the ACU lab. Following treatment, any remaining sample was made available for research but unlike the 'morning' samples it was not re-prepared before use (samples used in chapters 6&7).

Semen samples from patients attending the ACU are from couples attending for ART and are not necessarily all men with male factor infertility.

2.4 Sperm preparation

Semen samples from donors were prepared using the density gradient centrifugation (DGC) method to isolate spermatozoa from seminal plasma. A maximum of 1.5ml of semen was gently added to the top layer of the density gradient (2ml 40% Percoll® with an underlay of 2ml 80% Percoll®) and centrifuged at 300*g* for 20 min. The supernatant was discarded followed by collection of the 40% layer and 80% pellet into separate wash tubes. Samples were washed in 5ml NCM (centrifuged at 500*g* for 10 min) (Mortimer and Mortimer, 2013b). After centrifugation, the supernatant was discarded and the pellet was re-suspended in NCM or CM (source and catalogue numbers see appendix).

2.5 CASA parameters adopted in all experiments

Samples were diluted in NCM or CM to between $\geq 2x10^6$/ml (lower limit, too few cells for CASA to distinguish between debris and sperm cells resulting in an increased error) (Garrett et al., 2003) and $\leq 50x10^6$/ml (upper limit, measurement made above $50x10^6$/ml are at a higher risk of collisions increasing error) (WHO, 2010c) allowing accurate reading on the CASA. Unless otherwise stated, motility was measured after centrifugation at time 0 min (time 0 min = addition of NCM/ CM to sample and motility measured instantly) then every 30 min for 3 hrs. Samples were kept in the appropriate incubator (5% CO_2 or normal) between readings. The slides used for readings were 4

chambered 20µM depth slides that incur no known drift (MicroCell counting chambers, Vitrolife, Inc. San Diego, CA, USA).

A minimum of 200 cells were counted per frame, with a minimum of 2 frames counted per chamber, to give a minimum total of 800 motile spermatozoa counted (unless otherwise stated) in every experiment. This number was chosen in order to achieve an acceptably low sampling error when evaluating motile cells (WHO, 2010d).

2.6 Gradient experiment (Chapter 3) to determine most suitable fraction to be used as a surrogate for patients

Gradients were compiled from 1ml 80% Percoll® with an underlay of 1ml 90% Percoll®, followed by overlaying 2ml of 60%, 40% and 20% Percoll® (2ml of each added to reduce contamination between layers when collecting sample) with 1ml sperm added and then centrifuged at 300*g* for 20 min, as described previously. The supernatant was discarded followed by collection of each layer into separate wash tubes containing 5ml NCM. Each wash was centrifuged for 10 min at 500*g*. Samples were diluted in CM and readings taken at 0 min then every 60 min for 5 hrs. 400 cells counted per frame, 4 frames counted for each chamber giving a minimum total count of 1600 motile cells.

2.7 Assessment of calcium and motility induced by novel compounds

2.7.1 Flexstation Assay Screen (experiments conducted by Sarah Matins da Silva in combination with the Dundee Drug Discovery Unit (DDU))

Sperm samples were collected and prepared as previously described. Following preparation by DGC sperm cells were subjected to capacitating conditions for 2 hrs 30 min. Media and supernatant were removed and cells re-suspended in Flexstation assay buffer (1XHBSS, 20mM HEPES, 0.5mM probenecid, pH7.4) and concentration and volume ascertained. Approximately 110 million cells constituted in a 5ml volume were added to an equal volume of the Ca^{2+} sensitive dye, Calcium 3, which had been made up

to twice the manufacturer's recommended concentration. Following an incubation at 37ºC for 60 min, the sperm cells were recovered by centrifugation at 700*g* for 5 min at room temperature and re-suspended in Flexstation assay buffer at a concentration of 5 x 10^6 cells / ml. The cells were plated out onto 384 well clear bottom, black wall plates at a concentration of 2.5 x 10^5 cells / 50µl /well. The plates were then centrifuged at 700*g* for 5 min at room temperature to ensure the cells were at the base of each well. A plate was made up to contain test compounds at a 5x final concentration, with the use of 10µM progesterone as a control (plated by robotics at DDU). Both the assay and the plates containing compounds along with FLIPR tips were placed into the appropriate compartment of the Flexstation. The Flexstation recording was started and 12.5µl of test compound was added after 18 seconds (s). Ca^{2+} dependent fluorescence of the Calcium 3 dye was measured for 100s as recommended by the manufacturer (excitation wavelength = 485nm, emission wavelength = 525nm, cut-off = 515nm). Hit compounds were identified by software analysis of the area under the curve function when compared to progesterone (positive control). These compounds were then examined for any effect they may have on motility (see below).

2.7.2 Preparation for motility assessment of novel compounds

Following DGC preparation, each sample was diluted to a final concentration of 20million/ml. Samples were placed in appropriate incubators NCM-samples in normal (37^oC, 15 min), CM-samples in 5% CO_2 (37^oC, 2 hrs 30 min). 99µl of semen sample was then placed in labelled round bottom tubes with the addition of 1µl of appropriate drug. 1µl 1% DMSO was added to control tube as a vehicle control. 3µl of sperm suspension was then loaded onto preheated fixed chamber Microcell slides and CASA analysis conducted (described previously).

2.7.3 Sperm penetration into artificial viscous medium (Kremer test)

Kremer penetration tests were carried out using methylcellulose (an artificial mucus substitute) with a viscosity of 4000 cp (MC4000). This was prepared in NCM containing 0.3% BSA at 10mg/ml. As previously described (Ivic 2002), 5 cm long, rectangular, flattened capillary tubes with a depth of 0.4mm (Camlab Limited Cambridge UK) were placed into the methylecellulose for 30 min at 37^{o}C to allow the tubes to fill. Excess methylcellulose was gently wiped from the tubes and the upper ends of the capillary tubes sealed with plasticine. The open end of the tube was placed into the prepared sperm at a concentration of ~ 20x10^{6}/ml for donor samples. Patient samples were capacitated in the ACU lab for a minimum of 3 hrs (as described above) they were then diluted in clinic media to a concentration of ~ 2x10^{6}/ml. 1µl of compound was added to 99µl of sperm suspension, giving a final concentration in each case of 10µM A1 - E1, 40µM Trequinsin and 3.6µM progesterone. Controls contained 1% DMSO to account for any effects produced by DMSO itself. Sperm preparations and capillary tubes were then incubated in appropriate incubators (see above) for 1 hr. The capillary tubes were then removed and wiped to remove residual spermatozoa from the surface of the tubes. The open ends of the tubes were sealed with plasticine, and marks were made on the tubes at 1 cm and 2 cm. The tubes were then viewed on an Olympus CX41 microscope (20X objective final magnification x200) (Olympus Corporation, Tokyo, Japan), with the number of cells being counted at 1 and 2 cm distances from the base of the tubes (figure 2-1). Three fields of view were counted for three separate planes between the upper and lower wall of the capillary tube and an average obtained. Results were normalised to parallel untreated 1% DMSO controls to allow comparison between different experiments (source and catalogue numbers see appendix).

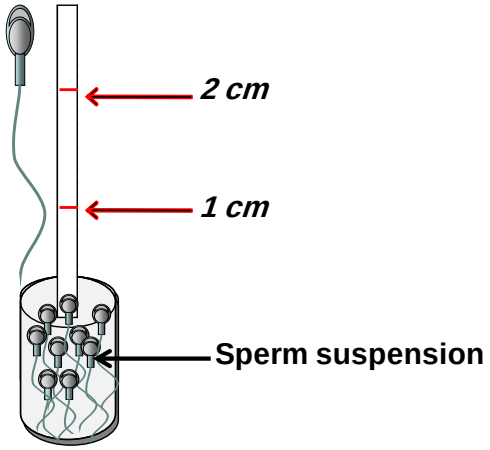


Figure 2-1 Model depicting sperm penetration into artificial viscous medium (Kremer test). Kremer capillary tubes filled with viscous medium were immersed in sperm suspensions in the presence or absence of compounds, and left at 37°C in a 5% CO_2 incubator for 1 hr to allow the migration of motile spermatozoa. Red lines on the tube show the locations at which sperm numbers were counted, i.e. 1 and 2cm from the base of the tube. (Alasmari, 2013)

2.8 Assessment of the Acrosome Reaction

Sperm obtained from donors was prepared by DGC as described previously. Sperm acrosomes were studied by staining with lectin from *Pisum sativum* (pea) (PSA) labelled FITC, as described by Liu et al (Liu and Baker, 1988). Prepared sperm were capacitated for 2 hr 30 min before being exposed to C1 or D1 at a final concentration of 10µM for 1 hr. A negative control was exposed only to CM (1 hr), a vehicle control was exposed to 1% DMSO (1 hr) and a positive control where sperm were induced to undergo the acrosome reaction using Ca^{2+} ionophore (A23187) at a final concentration of 10µM for 15 min (Aitken et al., 1993, WHO, 2010a). Sperm were washed twice with 10ml 0.9% sodium chloride by centrifugation at 600*g* for 10 min. The sperm pellets were re-suspended in 20-30µl sodium chloride and smeared on microscope slides, allowed to air dry. The smear was fixed in 95% (v/v) ethanol for 30 min at 4°C.

After some initial experiments to optimise conditions, the final protocol involved using 12.5µg/ml PSA labelled with FITC in phosphate buffered saline (PBS) (PSA (FITC)) for 1 hr at 4°C in the dark. The slides were washed in PBS three times and mounted

using hydromount (source and catalogue numbers see appendix). At least 200 sperm were counted from each slide with 2 slides per treatment, 400 sperm counted total per compound, on an EVOS xl digital inverted microscope (Advanced Microscopy Group) using fluorescence setting with 400x magnification. Images were captured on the EVOS fully integrated colour camera. When more than half a sperm head was brightly fluorescing the acrosome was considered to be normal and intact (figure 2-2)

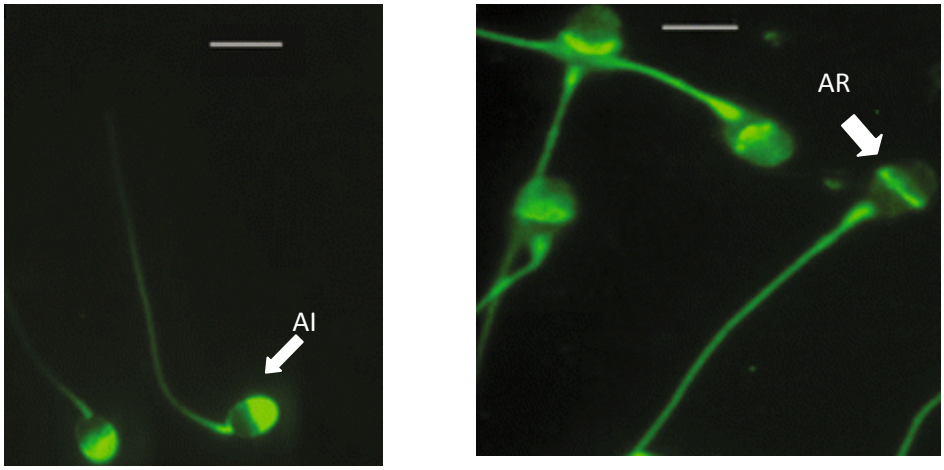


Figure 2-2. Image depicting acrosome intact (AI) and acrosome reacted (AR) spermatozoa. Acrosome intact spermatozoa were identified by more than half the sperm head brightly fluorescing whereas acrosome reacted spermatozoa were identified by having a fluorescing equatorial segment and no fluorescence elsewhere in the sperm head (Image courtesy of Jones et al.,2010).

2.9 Hypo-osmotic swelling (HOS) test

Agonists were examined to measure if they significantly compromised sperm viability by use of the hypo-osmotic swelling (HOS) test (Jeyendran et al., 1984). The HOS test measures sperm membrane integrity and for this test spermatozoa incubated with or without agonists were further incubated with hypo-osmotic medium (75 mmol D-fructose l^{-1} and 25 mmol sodium citrate dihydrate l^{-1}) at 37^{o}C in 5% CO_2 for 30 min (source and catalogue numbers see appendix). Sperm with coiled tails were counted as viable and were counted from 2 separate aliquots with a minimum of 200 cells counted per replicate to reduce sampling error.

2.10 Statistical analysis

Normality of data (the supposition that the underlying random variable of interest is distributed normally, or approximately so) was assessed according to frequency distributions and the Kolmogorov-Smirnov test. Results are expressed as the mean ± SD (standard deviation), mean ± SE (standard error). Statistical comparisons were made using T-test and the analysis of variance (ANOVA) if the data were either originally normally distributed or normalised after transformation. However, some data sets were not able to be normalised following transformation thus; the statistical analysis of this data was performed using non-parametric tests including Mann Whitney U test and Kruskal – Wallis one way analysis of variance on ranks. Pearsons Chi squared analysis was conducted when analysing results from the Hypo-osmotic swelling (HOS) test. Linear regression analysis was employed when analysing semen data from two separate clinics. Significance was considered to be $P \leq 0.05$. All statistical analysis was performed using SPSS statistics 18.

CHAPTER 3

Identification of a sub-group of donor spermatozoa after density gradient centrifugation that is comparable to a patient sample

3.1 Introduction

The availability of sub-fertile patient semen samples for research purposes is very limited and for this reason it would be invaluable to research to be able to isolate a sub-group of spermatozoa from donor samples that is similar to sub-fertile patients. Patient samples can be very variable having problems with low concentration, poor morphology, poor motility or combinations of these factors (Curi et al., 2003) making this task a difficult one. In an attempt to identify a 'surrogate' patient sample from normozoospermic donors density gradient centrifugation was used to separate donor samples into 6 fractions; 20%, 40%, 60%, 80% and 90%. Each fraction was then compared to the 80% fraction procured from sub-fertile patients for their motility characteristics. Motility is an important functional characteristic that is related to a spermatozoa's ability to successfully fertilise in IVF (Donnelly et al., 1998). Clear differences have been noted in sperm motility between sub-fertile and fertile men (Macleod and Gold, 1951b, Barratt et al., 2011) which corroborates the importance of identifying a surrogate population of donor sperm to be used in research if sub-fertile samples are not available. Poor sperm motility has been associated with defective mitochondrial DNA (Oaconnell, 2003) meaning that testing new drug therapies or preparation techniques on normozoospermic donors as a substitute for sub-fertile patients may give inaccurate results. To be able to accurately determine the effect of a new treatment on spermatozoa you need to be able to test on sperm that have similar qualities to that seen in patient samples.

DGC is currently the sperm preparation technique employed by assisted conception clinics for preparing spermatozoa before procedures. DGC was initially suggested to be used to enhance motility in cases of asthenozoospermia (Berger et al., 1985) and in the late 80's this technique was shown to enhance sperm penetration into human oocytes (Guerin et al., 1989). Previous studies have discovered that spermatozoa recovered from

different Percoll® fractions and incubated in capacitating media have the ability to maintain motility to different extends regarding which fraction the cells are recovered from (Saad and Guerin, 1992). Using this information this study attempted to isolate 6 different fractions (20%, 40%, 60%, 80% and 90%) from normozoospermic donor spermatozoa and compare the motility parameters from the spermatozoa recovered from each fraction to that of sub-fertile patient spermatozoa recovered from the 80% fraction. Previous studies have examined and analysed motility parameters from spermatozoa recovered from different fractions after DGC however, these studies were attempting to identify the fraction that contains the 'best' spermatozoa to be used clinically in ART (Saad and Guerin, 1992, Moohan and Lindsay, 1995, Forster et al., 1983, Yao et al., 1996). We have been unable to locate a study that compares fractions from donor spermatozoa with that of sub-fertile patients.

The main aim of this chapter is to isolate and identify a sub-group of spermatozoa from normozoospermic donors that has similar motility characteristics to spermatozoa recovered from patients undergoing fertility treatment.

3.2 Results

3.2.1 Comparison between sperm movement characteristics from normozoospermic donors after migration through 20%, 40%, 60%, 80% and 90% Percoll® gradients, with patient samples after an 80% Percoll gradient®

3.2.1.1 *Movement of spermatozoa recovered from different fractions of Percoll® (donor) compared to 80% fraction (patient)*

In the motility assay the motility parameters measured were VCL, ALH, LIN, total motile, progressive motile and hyperactivation for each fraction acquired from donor samples. This information was then compared to the information obtained for the same parameters from the 80% fraction of sub-fertile patients. In this study we are looking for the donor fraction that most closely reflects the motility parameters of the sub-fertile sample meaning we are looking at fractions where there is not a significant difference. The 20% fraction showed significant differences to the sub-fertile sample concerning VCL across all time points (0 min $P<0.01$, 60, 120, 240 and 300 min $P<0.05$) excepting after 180 min where no significant difference was noted (figure 3-1A and appendix). The 40% fraction was significantly decreased compared to the sub-fertile sample at 0 min ($P<0.05$) with no significant differences in VCL at any other time point (figure 3-1A and appendix). No significant difference was noted in the 60%, 80% and 90% fraction regarding VCL at 0, 120, 180, 240 or 300 min however, VCL was significantly increased in donor samples in all three fractions after 60 min (60%, 80% $P<0.05$, 90% $P< 0.01$) (figure 3-1A, and appendix).

Values for ALH were found to have no significant differences when compared to sub-fertile patient samples for any of the donor fractions analysed across the entirety of the 300 min time course (figure 3-1B and appendix).

Maximum linearity values were identified from spermatozoa recovered from the 60%, 80% and 90% fractions from donors and no significant difference was noted across the 300 min time course of each of these fractions when compared to the sub-fertile patient sample (figure 3-1C and appendix). The linearity values for the 20% and 40% fractions were similar (figure 3-1C) and significant decreases were found at time 0 min (20% *P<0.005,* 40% *P<0.05*), 60 min (20% *P<0.05)* and 180 min (20% and 40% *P<0.05)* in donor samples compared to patients (figure 3-1C and appendix).

The percentage of total motile spermatozoa recovered were maximal in the 60%, 80% and 90% donor fractions but owing to the huge variation in the percentage of total motile spermatozoa recovered from the sub-fertile patients. Significant increases in motility from donor spermatozoa were noted at time 0 and 60 min in the 90% fraction *(P<0.05)* compared to the 80% sub-fertile patient (figure 3-1D and appendix). The percentage of total motile in the 20% fraction were significantly lower than the sperm recovered from the sub-fertile patient sample at time 0 and 180 min *(P<0.05)* but no significant differences were identified at any of the other time points (60, 120, 240 or 300 min) (figure 3-1D and appendix). The 40% fraction showed no significant differences at any time points when compared to the sub-fertile sample (figure 3-1D and appendix).

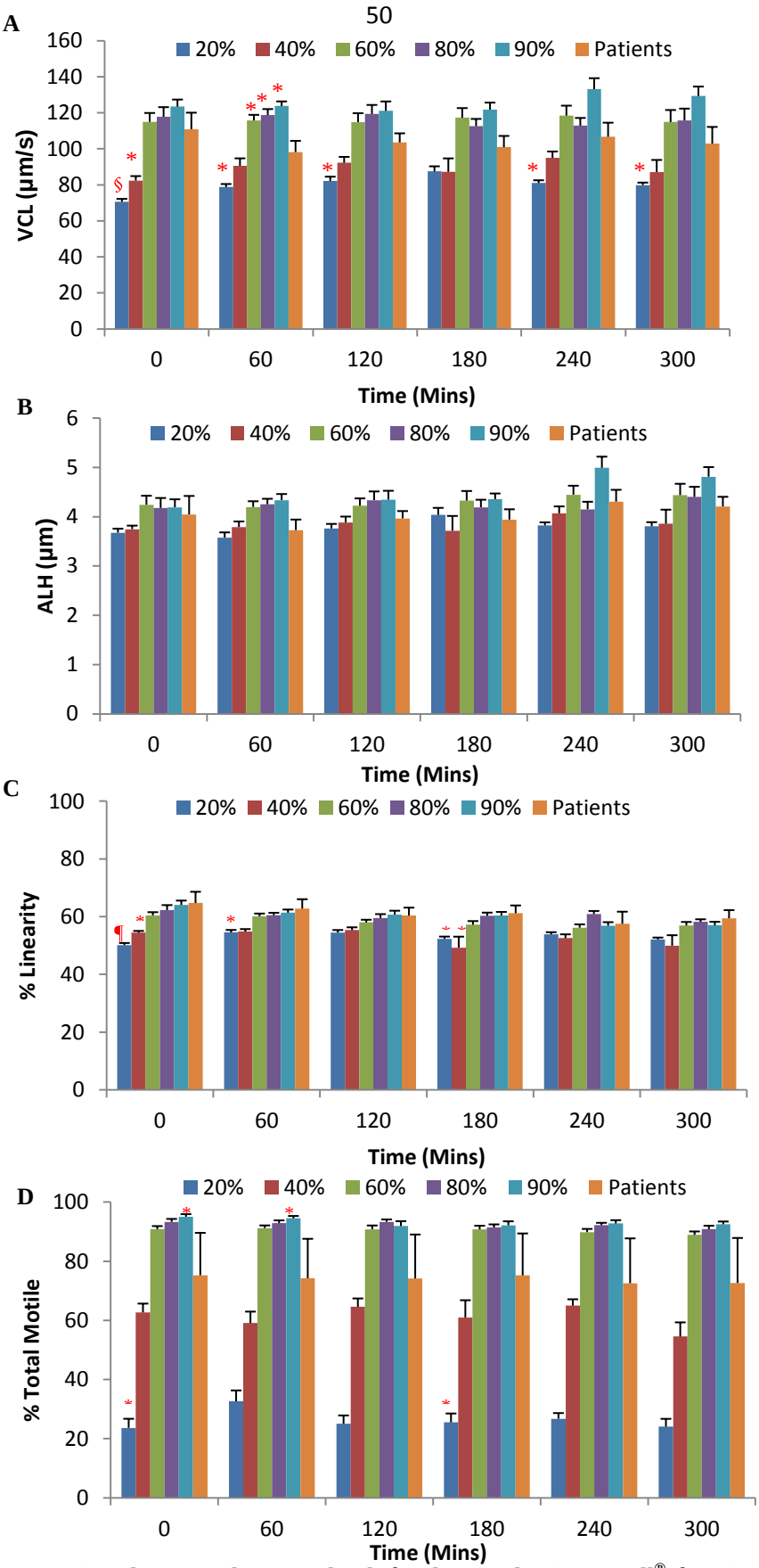


Figure 3-1 Comparison between donor and sub-fertile samples in Percoll® fractions relative to incubation time under capacitating conditions. Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction for VCL **(A),** ALH **(B),** LIN **(C)** and total motile **(D).** The result shown is the mean ± SEM. N= 5 for both donors and patients, * indicates significant difference (*P<0.05),*§ indicates *P<0.01,*¶ indicates *P<0.005* between each individual fraction and the patient sample (T-test and Kruskall-Wallace test).

The percentage progressively motile spermatozoa recovered from the 20% fraction were significantly lower than that of the sub-fertile patients at time 0 and 180 min *(P<0.05)* (figure 3-2A and appendix). Again there was a large variation in the percentage progressively motile cells recovered from the patient samples resulting in no significant difference being noted between the 40%, 60%, 80% or 90% fractions in comparison to the patient samples at any time point examined (figure 3-2A and appendix).

The percentage of hyperactivated spermatozoa was significantly lower in the 20% fraction at time 0 min in comparison to the sub-fertile sample *(P<0.05)* (figure 3-2B and appendix). A significant increase in the percentage of hyperactive spermatozoa was found in the 60%, 80% and 90% fractions after incubation for 60 min *(P<0.05)* compared to those recovered from the sub-fertile 80% fraction (figure 3-2B and appendix). There were no significant differences identified at any other time points (0, 120, 180, 240 or 300 min) for the 60%, 80% and 90% fractions. The 40% fraction showed no significant differences at any time point across the 300 min time course when compared to the sub-fertile samples (figure 3-2B and appendix).

Kruskall-Wallace statistical analysis showed that there was no significant difference in any of the parameters measured when looking independently at samples from 20%, 40%, 60%, 80%, 90% or patients across 300 min time course. This showed that incubation time didn't have an effect either positive or negative on the motility parameters measured (VCL, ALH, LIN, total motile, progressively motile and hyperactivation).

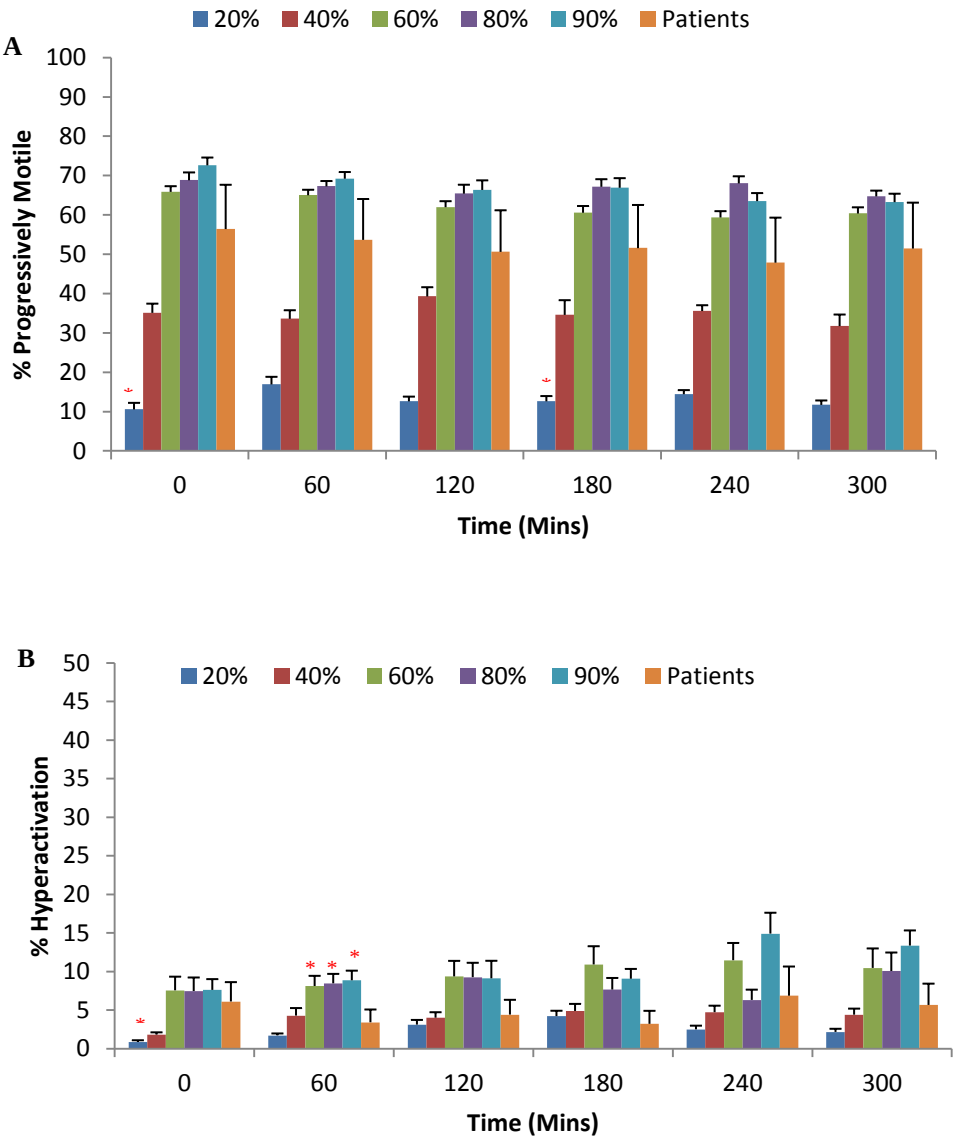


Figure 3-2 Comparison between donor and sub-fertile samples in Percoll® fractions relative to incubation time under capacitating conditions. Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction for progressively motile **(A)** and hyperactivation **(B).** The result shown is the mean ± SEM. N= 5 for both donors and patients, * indicates significant difference (*P<0.05)* between each individual fraction and the patient sample (T-test and Kruskall-Wallace test).

3.3. Discussion

Density gradient centrifugation is a useful sperm preparation technique that allows for collection of motile sperm by separation into separate fractions even in cases of asthenozoospermia (Berger et al., 1985). This technique is currently used clinically to obtain the 80% fraction containing the 'best' portion of spermatozoa to be used for fertilisation in artificial reproductive technology (Forster et al., 1983, Yao et al., 1996, Oaconnell, 2003). The data in this chapter corroborates that of other studies identifying that the proportion of spermatozoa collected after density gradient centrifugation from the higher percentage Percoll® fraction i.e. 90%, gives you the better overall motility parameters (Forster et al., 1983, Saad and Guerin, 1992, Yao et al., 1996). Although, the data in this study noted that there were no significant differences across any of the parameters measured between the 80% and 90% Percoll® gradient suggesting that an 80% gradient is of a high enough density to retrieve highly motile spermatozoa. In this study the patient samples did undergo a second Percoll® gradient before comparison with the donor samples. This could have had a detrimental effect on the motility of these samples however previous work in our laboratory has suggested that the effects of a second Percoll® gradient are negligible on motility. Previous studies available have compared motility parameters (Berger et al., 1985, Forster et al., 1983, Moohan and Lindsay, 1995, Saad and Guerin, 1992), morphology (Yao et al., 1996, Forster et al., 1983), vitality, interaction with zona-free hamster eggs (Forster et al., 1983) and DNA damage (Oaconnell, 2003, Ghaleno et al., 2013) between differing fractions obtained after density gradient centrifugation from the same sample, however, we have been unable to identify any studies that compare fractions taken from normozoospermic donors with sub-fertile patient samples.

The results from this study show that, concerning VCL, the variability in the patient samples means it is not possible to discern which fraction is most similar with 40%, 60%, 80% and 90% all having values similar to that of the patient sample. With regard to ALH, it was again not possible to determine which fraction was comparable to sub-fertile samples with all fractions expressing similar values (including patients) reflecting findings of previous groups (Saad and Guerin, 1992). The data presented in this chapter identifies significant differences in more than one parameter and at more than one time point for both the 20% and 90% donor fractions when compared to the 80% sub-fertile fraction suggesting that they are not suitable comparable fractions to patient samples. The 40%, 60% and 80% fractions are all showing similarities to the 80% sub-fertile patient samples and from the data available in this study it is not possible to determine which of these three fractions is most similar to patients. However, in this study it was decided to use the 40% fraction as our poor motility group.

This study has looked specifically and solely at motility parameters and we are aware that to be able to accurately determine a sub-population of donor spermatozoa that is similar to that of sub-fertile patients, further in-depth analysis would need to be completed looking at morphology, DNA damage, vitality, penetration ability, acrosome reaction and also account for bacteria/debris (Aitken et al., 1995). This may be the reason that this study has not been attempted previously due to the many variables that need to be accounted for to make it a valid study. Another factor that may discourage people from completing this study could be the knowledge that variation between patient samples is large (Macleod and Gold, 1951b) and the ability to identify any useful data would depend on having a large sample size, which can be difficult to obtain.

CHAPTER 4

Development of Standard Operating Procedure for Drug Screening

4.1 Introduction

In vitro and *in vivo* sperm motility can be affected by a variety of factors some of which can be more easily controlled in an *in vitro* environment such as; temperature (37^{o}C) (Esfandiari et al., 2002, WHO, 2010f), exposure to non-capacitating or capacitating conditions and types of apparatus used. As an attempt to increase the repeatability and reliability of any results obtained within this project, preliminary investigations were performed and environmental factors systematically examined thereby producing a protocol that limits/accounts for variation in results.

The focus of this chapter was to examine sperm motility under a variety of conditions addressing six key questions:

1. Given the variability within a prepared human sperm population, is it more accurate to assess motility parameters in 200 cells from 4 different aliquots compared to 400 cells from 2 different aliquots?
2. Does the use of slides with fixed coverslips give a more accurate representation of motility when compared to reusable slides and coverslips?
3. What is the effect of NCM and CM on sperm motility?
4. Do CM and NCM have a detrimental effect on sperm motility when incubated at 37^{o}C for long periods of time?
5. Are motility responses consistent within and between ejaculates?
6. Does 1% DMSO have a detrimental effect on sperm motility under non-capacitating and/or capacitating conditions?

4.1.1 Minimum number of cells counted to reduce sampling error

Spermatozoa are erratic and changeable and therefore huge variation in motility within a single sample can occur. Standard procedure describing analysis of motility parameters using CASA recommends that a minimum of 200 cells per frame from each replicate is measured to reduce sampling error (WHO, 2010d, Fraser and Group, 1998). However, it is not only important to identify a reliable method of evaluating motility, but to do so in the most economical way. To determine whether it was possible to read more than 200 cells from one replicate whilst maintaining similar variability within results, an investigation was devised using a sample split into two groups: 1) four separate aliquots of 200 cells measured per time-point and 2) two separate aliquots where 400 cells were measured per time-point (see chapter 2). Semen samples were prepared in the same way and cells diluted to the same concentration.

4.1.2 Variation in motility assessed by different slide types

When assessing sperm parameters using CASA, there is large variation in sperm concentration and motility according to slide type used (Tomlinson et al., 2001). A comparison of CASA motility parameters was performed to assess differences between the two slide types available in our lab, which could account for variation within results, 2xCell Hamilton Thorne slides (20µm) with separate coverslips (figure 4a) and Microcell 4 fixed chamber slides (20µm) (figure 4b) were evaluated. The Hamilton Thorne slides are reusable and therefore washed and recycled, whereas the Microcell slides are disposable, single use only.

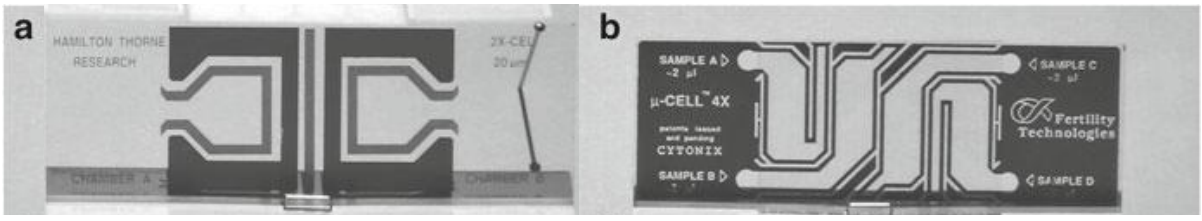


Figure 4 Examples of slide types examined. (a) 2xcell Hamilton Thorne slide with manual cover-slips (b) Microcell 4 fixed chambered slides. Image adapted from Spermatogenesis (Mortimer and Mortimer, 2013b).

4µl of prepared sperm was added to each chamber of the Hamilton Thorne slides and coverslips overlaid thereafter, whereas 3µl per chamber was added to the Microcell fixed chamber slides. A time-course experiment was run using a control sample in 1% DMSO. Samples were split and motility measured on each different slide type and results analysed. The hypothesis was that the Hamilton Thorne slides with the coverslips that are manually placed would have an increased chance of causing cells to stick to the slide resulting in a decreased total motility and progressive motility compared to the fixed chamber slides.

4.1.3 Between-donor and within- donor biological variation

If a series of samples are taken from the same person for semen analysis, the results of each of the independent deposits will not be exactly the same (Alvarez, 2003). The results may vary over time due to a variety of factors including; sexual abstinence period, transport of the sample to the laboratory and inherent biological variation (Alvarez, 2003). Spermatogenesis in mammals can range from 30 to 78 days and in humans the process takes over 70 days (Hess and Renato de Franca, 2008). To be able to account for any within-donor variability as a result of variation in spermatogenesis, repeat experiments were performed to compare the same sample over a 4 month period. Samples were subjected to capacitating conditions (2 hr 30 min, 37^{o}C, 5% CO_2) before motility assessments (CASA) were performed. Each sample was subjected to the same conditions to reduce variation from the external environment.

When the same tests are performed on different individuals the results once again would not be the same (Alvarez, 2003). In order to obtain robust and reliable results it is important to also be aware of between-donor variability. To be able to account for this a comparison in motility parameters between the same 5 donors examined for within-donor variability was conducted.

4.1.4 Effect of DMSO on sperm motility

DMSO is a solvent used to solubilise and stabilise compounds generated by UoD DDU. In future experiments we planned to examine motility effects of DDU library compounds, and therefore investigated the effects of DMSO on human sperm. All DDU compounds were provided in solution in 100% DMSO, and these were further diluted to concentrations of 4mM or 1mM respectively (chapter 2). These prepared stocks were diluted 1:100 with the prepared sperm sample making final concentration of DMSO in solution no more than 1% (known non-toxic concentration (de Lamirande and Gagnon, 2002)). To ensure that 1% DMSO had no detrimental effect over time, serial motility assessments were made and compared with control (either NCM or CM alone) over a 3hr time course.

4.2 Results

4.2.1 Development of methods for motility assessment by CASA

4.2.1.1 *Determination of the number of aliquots from a single sample that need to be taken to assess motility parameters*

To determine the number of aliquots that need to be taken from a single sample in order to reliably assess motility parameters, a total of 800 motile cell counts were taken from either two aliquots from the same sample or four aliquots from the same sample. Motility parameters were measured from 40% and 80% fractions under non-capacitating and capacitating conditions to allow for any variation between fractions and types of media used. Figure 4-2 A&B shows the range for 800 motile cell counts in NCM and CM for 40% and 80% fractions. The range of results for reading cells from only two aliquots compared to four aliquots are very similar for both total motility and progressive motility (appendix). There is also no clear difference seen in ranges of total and progressive motility when reading 800 motile cells following incubation under capacitating conditions in either 40% or 80% fractions (figure 4-2 C&D and appendix). Results recorded for all variables also indicate that there is no statistical difference in range of all parameters measured at all time points (VCL, ALH and hyperactivation (appendix)).

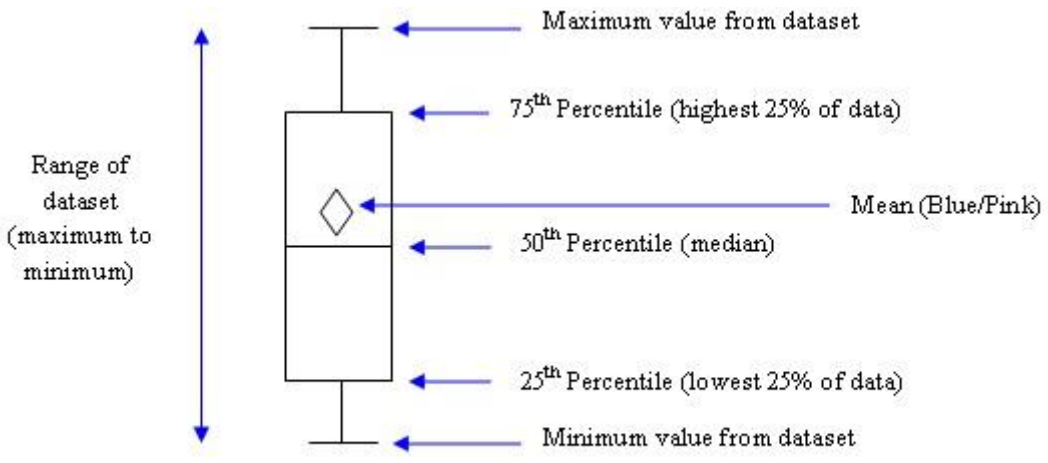


Figure 4-1 Key for identifying features in Box Plots (figures 4-2 & appendix).

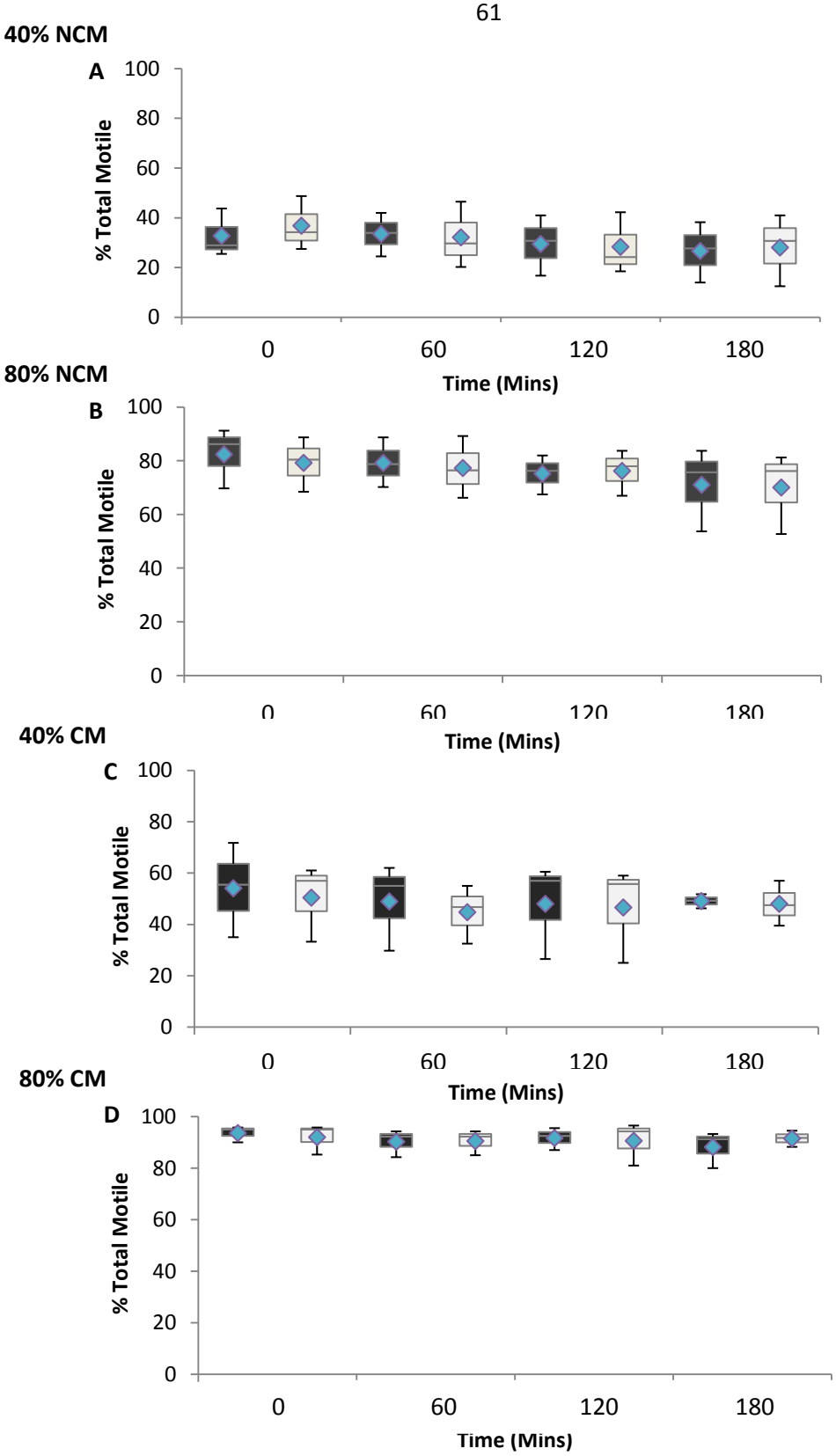


Figure 4-2. Variation in range of results 4 aliquots vs 2 aliquots in non capacitating and capacitating buffer for % total motile (A) 40% NCM **(B)** 80% NCM **(C) 4**0% CM **(D)** 80% CM. The result shown is the mean (blue diamond) ± the range for A-D, see **figure 4-1** for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

4.2.1.2 *Determination of slide type required for CASA assessment of motility*

Hamilton Thorne 2X-Cell chambered slides (manual coverslips) were compared to MicroCell fixed counting chamber slides. Prepared sperm (80% fraction) were subjected to capacitating conditions and motility parameters, (VCL, ALH, hyperactivation (appendix), total motile and progressive motility (figure 4-3 A&B)), subsequently measured using CASA. 800 cells were counted for both slide types. Data from repeat experiments (n=3) was pooled, and results statistically analysed using Mann Whitney and Kruskall Wallace tests. Progressive motility was significantly increased in samples on the MicroCell fixed counting chamber slides compared to the Hamilton Thorne manual slides at both 60 min ($P \leq 0.05$) and 120 min ($P \leq 0.05$) (figure 4-3 B).

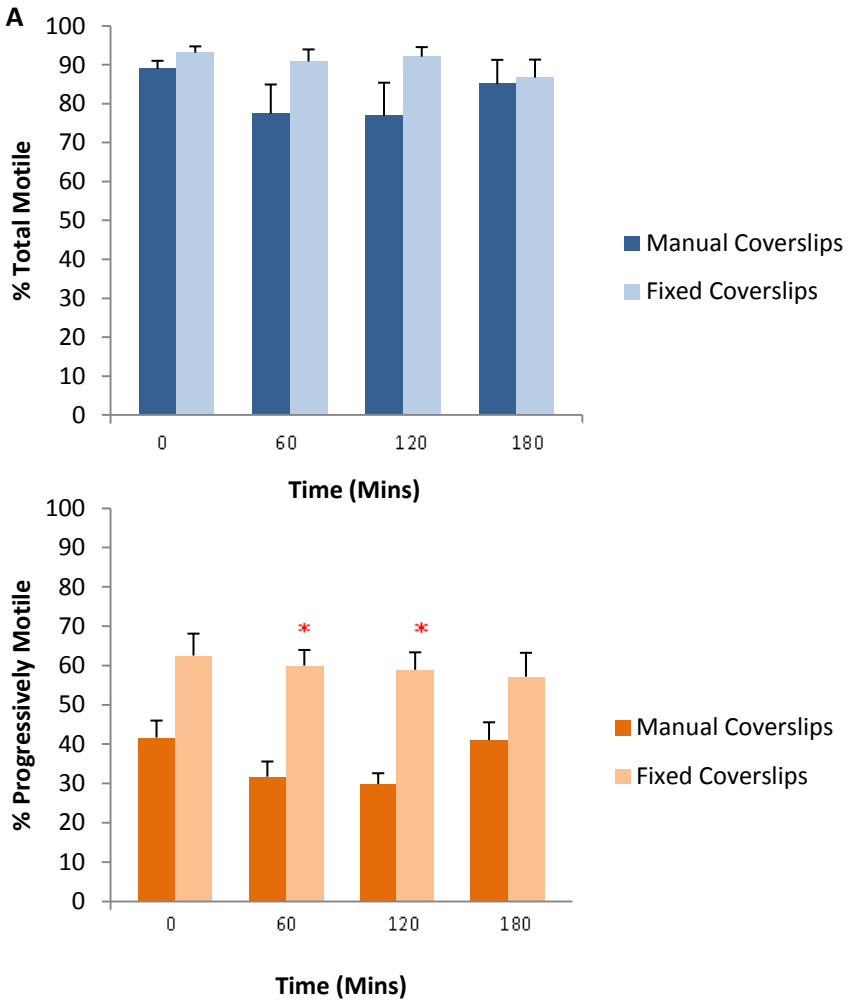


Figure 4-3. Variation in motility between two slide types (A) % total motile **(B)** % progressively motile. The result shown is the mean ± SE measured at defined time points up to 180 min N= 3 for both parameters, * indicates a significant increase between manual and fixed slides at independent time points. Significance was considered as $P \leq 0.05$

4.2.1.3. *Assessment of motility parameters in capacitating and non-capacitating media*

To determine optimal incubation times under capacitating and non-capacitating conditions, prior to acquiring CASA readings, motility parameters were measured over a period of 180 min in both NCM and CM. As expected, in the 40% fraction there was a highly significant ($P \leq 0.01$) increase across all motility parameters measured (VCL, ALH (appendix), total motile, progressively motile and hyperactivation (figures 4-4 A,C,E)) for samples incubated in CM compared to NCM across all time points. Similarly, the 80% fraction showed highly significant increases in VCL ($P \leq 0.001$) and ALH ($P \leq 0.05$) in CM compared to NCM across all time points (appendix). Hyperactivation also showed highly significant increases ($P \leq 0.001$) at time 0, 60 and 120 min, with a $P \leq 0.005$ at 180 min. There was no significant difference seen in total motility between the two media types with progressive motility only being significantly increased after 180 min ($P \leq 0.005$) (Figures 4-4 B, D, F). Kruskall-Wallace statistical analysis showed that there was no significant difference in any parameters measured when looking independently at samples in NCM or CM (40% and 80% fractions) across 180 min time course.

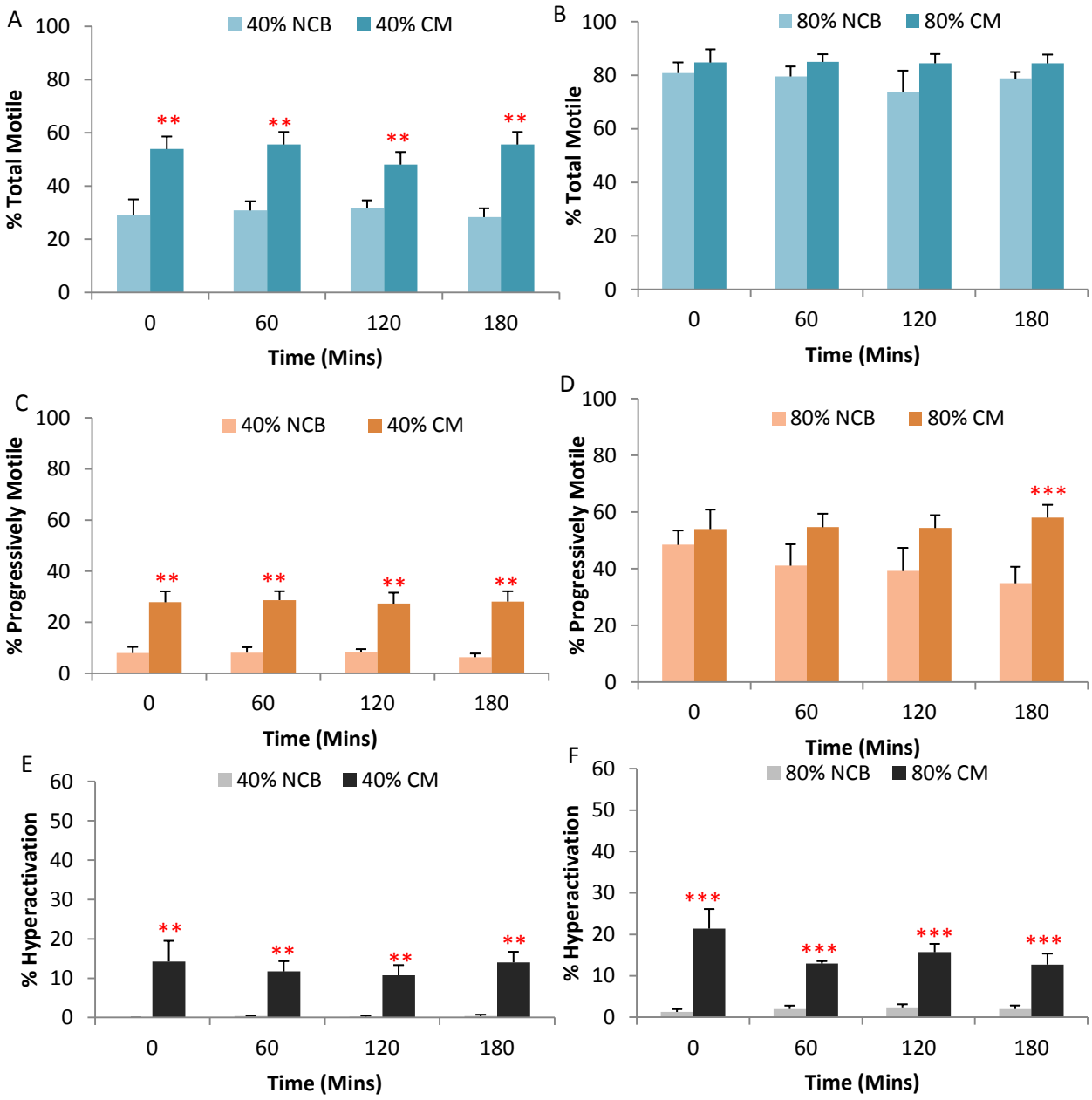


Figure 4-4. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating media (CM) for 40% and 80% fractions. % total motile: **(A)** 40% **(B)** 80%, progressively motile: **(C)** 40% **(D)** 80% and hyperactivation: **(E)** 40% **(F)** 80%. The result shown is the mean ± SE for % total motile, % progressively motile and hyperactivation. In this study all motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the difference between the two media types. N= 6 for both parameters for the 40% fraction and N= 5 for NCM and n= 9 for CM for the 80% fraction, ** indicates a significant increase ($P \leq 0.01$), *** indicates a significant increase ($P \leq 0.005$ or $P \leq 0.001$) between NCM and CM at independent time points (Mann Whitney test and Kruskal-Wallace test).

4.2.2 Development of methods for the assessment of motility for drug screening

4.2.2.1. *Inter-ejaculate and Intra-ejaculate variation with respect to motility parameters measured on CASA*

In order to examine the variability in motility between different ejaculates from the same man, the percentage of motile cells and progressively motile cells were examined from 5 donors on two separate occasions. When evaluating within donor variation 2/5 donors showed similar motility in both ejaculates (no significant differences) (highlighted in figure 4-5A) however, 3/5 donors showed a significant increase ($P \leq 0.05$) in the percentage of motile cells in the 2nd sample compared to the 1st sample assessed (figure 4-5A). With regard to progressive motility 2/5 donors showed significant differences ($P \leq 0.05$) between the 2 samples produced with the remaining 3 donors indicating no significant changes (figure 4-5B).

As expected, the variation between donor samples was found to be greater than the within sample variation. The percentage of motile cells was found to be significantly different ($P \leq 0.05$) between every donor, with the exception of donor 1 (d1) in comparison to donor 2 (d2) and d2 in comparison to donor 5 (d5) (highlighted in figure 4-5A), when comparing the 1st sample from each donor. However, when evaluating the difference between the 2nd samples produced the only significant result was d1 which was significantly decreased ($P \leq 0.05$) compared to the other 4 donors. In the case of progressively motile cells the 1st samples were all significantly different ($P \leq 0.05$) to one another except in one instance, donor 4 (d4) compared to d5 (highlighted in figure 4-5B). The variation in the 2nd sample was not as great as the 1st sample with donors showing similar (non-significant) results (d1 compared to d3, d1 compared to d4 & d2 compared to d5). The remaining samples were significantly different to one another (figure 4-5B). The results from this study highlight the variability in assessments, both, within the same donor's ejaculate and between different donor samples.

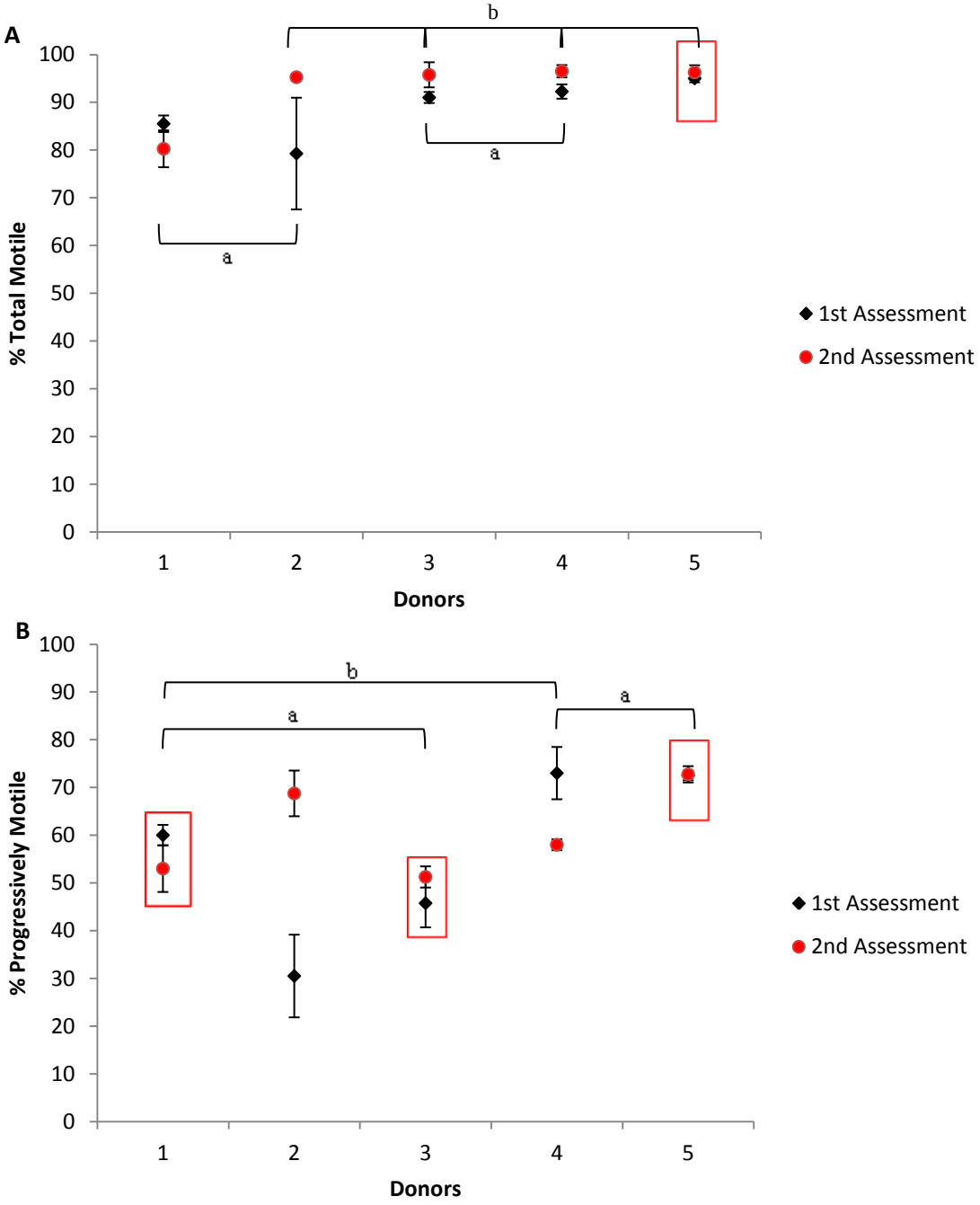


Figure 4-5. Inter-ejaculate variation in 5 donors following two assessments (A) % basal total motility **(B)** % basal progressive motility. The result shown is the mean ± SD for % total motile and % progressively motile. In this study, motility was assessed twice in 5 donors over a 4 month ± 1 week period. When evaluating **within** donor variation, where there is **NO** significant differences between the 2 assessments the result is highlighted with red boxes. When evaluating **between** donor samples 'a' denotes **NO** significant difference when comparing samples produced for the **1st** assessment, 'b' denotes **NO** significant difference between donor samples when comparing samples produced for the **2nd** assessment.

4.2.2.2. *Assessment of motility parameters in capacitating and non-capacitating media treated with 1% DMSO*

Compounds identified through HTS were prepared in solution with ≤1% DMSO. To determine if 1% DMSO is detrimental to motility parameters, a sample was treated under capacitating and non- capacitating conditions ± 1% DMSO for 180 min. This investigation enabled any adverse or influential reaction seen in sperm motility as a result of 1% DMSO treatment to be excluded. No significant difference was noted in any of the motility parameters measured (VCL, hyperactivation, total motile and progressively motile (figure 4-6 A-D and appendix)). The lack of significance, as analysed by Mann Whitney and Kruskall-Wallace tests, in both 40% (appendix) and 80% fractions (figure 4-6 and appendix) , in all media types examined (CM and NCM ± 1% DMSO), indicates that the addition of 1% DMSO to media does not impact on motility throughout 180 min incubation.

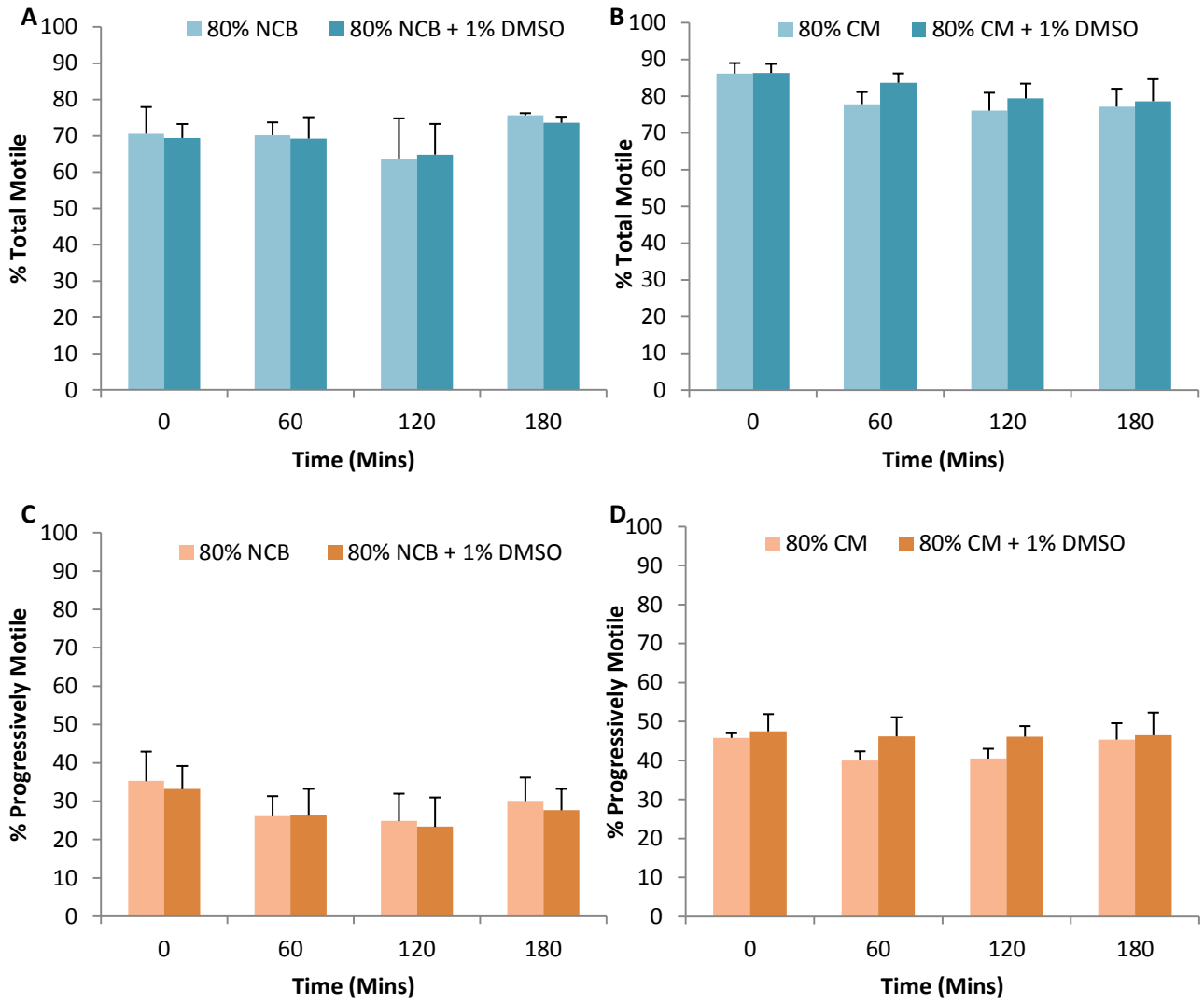


Figure 4-6. Expression of basal (control) motility values in non-capacitating (NCM) and capacitating (CM) media treated with 1% DMSO for 80% fraction (A) % total motile NCM, **(B)** % progressively motile NCM, **(C)** % total motile CM and **(D)** % progressively motile CM. The result shown is the mean ± SE for % total motile and % progressively motile. In this study all motility parameters were measured over a period of 180 min under non capacitating and capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 3 for NCM and NCM + 1% DMSO, N= 4 for CM and CM + 1% DMSO, no significant difference noted at any time point.

4.3 Discussion

The aim of these experiments were to examine the potential variables in the protocol proposed for screening sperm samples from donors and sub-fertile patients in order to improve experimental efficiency and to standardise laboratory protocol.

4.3.1 Repeatability and Reliability of system

4.3.1.1 *Number of aliquots from a single sample that need to be taken to assess motility parameters*

Sample variation within an individual occurs as a natural part of any semen sample (Fraser, 2001, Alvarez, 2003). The reasons behind this variation include period of sexual abstinence, transport of the sample to the laboratory, analytical and systematic errors (CASA) and inherent biological variation (Fraser, 2001, Alvarez, 2003). Sperm preparation methods are designed to isolate high grade spermatozoa from low grade spermatozoa and debris (Mortimer, 2000a, Mortimer and Mortimer, 2013a) and thus reduce sample variation within a single sample, although these methods do not completely eliminate it. Individual samples contain inherent random fluctuations around a homeostatic set point which can be influenced by environmental and biological factors (Fraser, 2001), and not all ejaculates from the same individual or between different individuals will therefore be identical. This variation needs to be recognised and accounted for if comparisons within populations or between clinics are to be made (Fraser, 2001, Alvarez, 2003, Jørgensen et al., 2001, Castilla et al., 2006).

Furthermore, the limitations of CASA technology must also be considered. Although CASA systems may be discredited as being poor determinates of semen concentration, they allow objective analysis of sperm motility kinematics as long as standard operating procedures are followed (Fraser, 2001, Mortimer and Mortimer, 2013a). The effect of sampling error on sperm concentration has previously been examined using motile

human sperm, analyzing a minimum of 200, 400, and 1,000 sperm (Tomlinson et al., 2010). Results were found to be similar regardless of the number of sperm assessed by this group (Tomlinson et al., 2010). To exclude variation as a result of the use of CASA this study followed expert recommendations on how to use CASA, limiting errors to a minimum (Fraser, 2001, Tomlinson et al., 2010, Mortimer and Mortimer, 2013a).

The standard procedure, as defined by ESHRE, states that at least 200 motile spermatozoa should be analysed per sample/aliquot when determining sperm motility characteristics using CASA (Fraser and Group, 1998, Tomlinson et al., 2010). Inaccuracies can occur as a result of in homogeneity errors when using fixed chamber slides (Mahmoud et al., 1997) where the use of a smaller volume i.e. 6µl (2 aliquots) may not be as representative of a whole sample when compared to 12µl (4 aliquots) (Mahmoud et al., 1997, Tomlinson et al., 2001). This study has shown that if the sample is thoroughly mixed (flicking of the tube to re-suspend cells) before analysis, there is no significant difference in the variation in the sample whether you read 4 separate aliquots or 2 separate aliquots (figure 4-2). Under capacitating conditions the range in variation of the results in the 80% fraction is considerably narrower than that seen in the 40% fraction in both systems. This is as expected, because the 80% fraction contains a high proportion of motile and progressively motile spermatozoa (Oaconnell, 2003, Mortimer and Mortimer, 2013c). Overall, the results of these experiments do not show any clear difference between the two approaches to CASA analysis when assessing cells under either capacitating or non-capacitating conditions (40% or 80% fractions), indicating that either approach could be used with minimal sampling error. As the more economical of the two systems it was decided to use the 2 replicate, 400 cells per replicate system in the final protocol.

4.3.1.2 *Determination of slide type required for assessment of motility*

Variation in concentration between the two slide types was not measured in this study although it has previously been examined (Mahmoud et al., 1997, Tomlinson et al., 2001). The reusable Hamilton Thorne slides have been shown to overestimate sample concentration as cells may not spread evenly across the chamber (Mahmoud et al., 1997, Hoogewijs et al., 2012). This could impact on cell motility by 1) increasing cell clumping and 2) increasing the chance of adherence of cell to slides, despite the presence of BSA in the media (Mortimer and Mortimer, 2013a). Hamilton Thorne slides may be treated with a nitrocellulose and polyvinyl coating to prevent sperm adhesion and to decrease effects on motility (Chapeau and Gagnon, 1987) although, it is not advisable to treat fixed chamber slides in the same manner. Another factor that may contribute to sticking when using Hamilton Thorne slides includes frequent washing, slides are washed using hot tap water and vigorous rubbing, they are then rinsed with deionised water and allowed to air dry in an upright position. This process can result in smears and scratches along with other superficial damage to the slides and/or coverslips which can alter motility itself and increase mistakes when analysing on CASA. Both slide types use a small volume (3µl or 4µl for disposable and reusable slides respectively) which could explain some of the variability seen in the results (figure 4-3 A-B). However, samples were mixed before addition to slides to minimise variation. Variability (Mahmoud et al., 1997) seen on the reusable slides could be due to differences between chambers, presumably as a result of unequal pressure being asserted by the individual placement of coverslips. The two slide types used in this investigation are also loaded in different manners, the Hamilton Thorne 2xCell slides with separate glass coverslips are filled by ‘drop loading’ whereas the MicroCell 4 chambered fixed coverslips are filled by capillary loading (Mahmoud et al., 1997, Mortimer and Mortimer, 2013a). Fixed chamber slides that are filled by capillary flow

are subject to the Segre Silberberg effect (Douglas-Hamilton et al., 2005a, Douglas-Hamilton et al., 2005b, Leja, 2005, Mortimer and Mortimer, 2013c). Although the Segre Silberberg effect has been proven to affect concentration readings it has very little bearing on motility and thus would have minimal effect in this study (Mortimer and Mortimer, 2013c). It is known that slide types with manual coverslips incur drift and should to be left to equilibrate for 60secs before recording results (Mortimer and Mortimer, 2013c). The MicroCell 4 fixed chambered slides incur no known drift and readings can be taken instantly after addition of sample.

Disposable, single use, fixed chamber slides are becoming the preferred choice for many IVF laboratories (Tomlinson et al., 2001). They can be used to measure concentration and motility and have been shown to produce reproducible and consistently reliable counts when examined using latex beads and homogenised sperm samples (Mahmoud et al., 1997). There are clear advantages to using fixed chamber slides with consistent depths of 20µm for assessing sperm motility when compared to standard reusable glass slides with separate cover slips (Tomlinson et al., 2001) especially as sperm motion characteristics are heavily dependent on chamber material and depth (Le Lannou et al., 1992, Kraemer et al., 1998). In contradiction to this study, Tomlinson et al (2001) previously measured motility between reusable glass slides (Makler and Horwell) and fixed chambered slides (Cell Vu, MicroCell and Leja) with no significant differences noted in motility parameters. This conflict may be explained by methodological differences. As a result of this experiment, unless unavailable, the MicroCell 4 fixed chamber slides were used preferentially.

4.3.1.3 *Assessment of motility parameters in capacitating and non-capacitating media*

Following ejaculation, capacitation (a complex series of molecular events that sperm undergo) occurs allowing sperm to be able to fertilise oocytes (Visconti et al., 2002).

Importantly this process can be replicated *in vitro* in specialised culture media, the composition of which is based on electrolyte concentration of oviductal fluid (Visconti et al., 2002). To enable capacitation to occur, capacitating media contains energy substrates, such as pyruvate, lactate and glucose, a cholesterol acceptor (serum albumin), $NaHCO_3$, Ca^{2+} , low K^+ , and physiological Na^+ concentrations (Visconti et al., 2002). The key component that differs between the two media types used in this study (NCM and CM) is the addition of HCO_3^- to CM. HCO_3^- is essential for the development of capacitation and sperm motility (Boatman and Robbins, 1991a, Visconti et al., 1995a). An increased intracellular HCO_3^- and Ca^{2+} stimulate soluble adenylate cyclase activity in sperm which leads to an increase in tyrosine phosphorylation resulting in increased motility and hyperactivation (Visconti et al., 2002, Visconti et al., 1995a, Battistone et al., 2013). This explains the significant increase in all motility parameters measured (VAP, VSL, VCL, total motile, progressively motile and HA) in the 40% fraction across all time points and similarly (VCL, ALH, progressively motile (180 min) and hyperactivation) in the 80% fraction when samples are in CM compared to NCM (appendix).

Sperm velocity is highly temperature dependent and it is essential that experiments be carried out at 37^{o}C to attempt to obtain as close to the physiological environment as possible (Appell and Evans, 1977, Mortimer and Mortimer, 2013c). The samples in NCM were incubated for 15 min after preparation (DGC) before readings were taken to allow the sample to return to 37^{o}C before analysis. The cells in CM were incubated for 2 hrs 30 min at 37^{o}C with 5% CO_2 to allow capacitation before analysis. Motility parameters remained stable for both fractions in either media, over the entire period of analysis (180 min). This indicates that the spermatozoa were stable in both media types over a period of 3 hrs at 37^{o}C. As a result of capacitation, there was a difference in the incubation times between samples in NCM and CM before motility analysis, however

both media results show no significant deterioration over time suggesting that no significant difference in the results would be seen for NCM samples if they had been incubated for 2 hrs 30 min in advance of analysis.

4.3.1.4 *Determination of variability between ejaculates from the same donor and different donor samples*

Numerous studies have been published discussing the biological variation in semen parameters (Mallidis et al., 1991, Alvarez, 2003, Keel, 2006). The smallest between subject variability in this study was found for total motility which is in accordance with previous studies (Poland et al., 1985, Alvarez, 2003). The smallest within subject variability in this study was found for progressive motility which differs to previous studies (Poland et al., 1985, Alvarez, 2003) however, it has been previously reported that variations in semen parameters produce contradictory results between groups (Mallidis et al., 1991). The within subject variability in this study showed lower levels of variation than the between subjects variation which is in agreement with previous reports (Heuchel et al., 1983, Alvarez, 2003). The results from this study, although evaluating a limited number of donors, corroborates previous reports and reinforces the need to be aware of variation both within subjects and between donor samples when conducting experiments. Thus, in chapter 5, 6 and 7 where some donors were assessed on more than one occasion, each sample was treated independently.

4.3.1.5 *Assessment of motility parameters in capacitating and non-capacitating media treated with 1% DMSO*

Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent (Rammler and Zaffaroni, 1967). Many compounds, therapeutic and toxic, that are not soluble in water are often soluble in DMSO. The physiologic and pharmacologic properties of DMSO are not completely understood (Brayton, 1986). DMSO is utilised in many biological

applications because it has many properties that are important for use, including rapid and enhanced penetration of other substances across biologic membranes; free radical scavenging; effects on coagulation and anticholinesterase activity. The systemic toxicity of DMSO is considered to be low making it suitable for addition to biological compounds (Brayton, 1986). The majority of HTS in drug discovery programmes use DMSO stock solutions because compounds are both highly soluble in DMSO and conferred long-term stability by its presence. However, to avoid adverse solubility enhancement of compounds and to reduce potential co-solvent effects, it is generally recommended that DMSO concentrations be kept to a minimum of ≤1% (Alsenz and Kansy, 2007).

It has been reported that media not exceeding 1% has no detrimental effect on sperm motility, capacitation or the acrosome reaction (de Lamirande and Gagnon, 2002). However, there is very little other information available describing the effect of DMSO alone on sperm, although in research it is readily used in combination with compounds added to sperm suspensions (de Lamirande and Gagnon, 2002, Lefievre et al., 2002, Marín-Briggiler et al., 2005, Agirregoitia et al., 2010b, Wang et al., 2013). The effect of DMSO on sperm motility was therefore examined, in order to account for any favourable or adverse reactions as a result of its presence if added whilst screening drug discovery compounds. No significant differences were seen in any of the motility parameters measured in any sample in either media with or without 1% DMSO over a 180min time course. This result corroborates previous studies, showing that presence of 1% DMSO has no effect on human sperm motility.

CHAPTER 5

Motility Analysis of Sperm treated with Hit Compounds identified through a Flexstation High Throughput Screening Assay

5.1 Introduction

Store operated Ca^{2+} channels at the neck/midpiece region of spermatozoa, through a CICR mechanism, have been identified as being regulators of sperm motility (Harper et al., 2004, Costello et al., 2009) along with the discovery of the sperm specific CatSper channel which is primarily associated with the development of hyperactivated motility triggered by depolarization- induced Ca^{2+} entry (Ren et al., 2001, Krasznai et al., 2006, Barratt, 2011). CatSper has enormous potential as a target for pharmaceutical manipulation due to its unique expression in sperm (Martins da Silva et al., 2012).

Martins de Silva *et al* developed a high throughput screening assay that identified compounds that increase $[Ca^{2+}]_i$ in human sperm (Martins da Silva et al., 2012). Compounds from this high throughput assay screen were identified, using Ca^{2+} as a surrogate for motility, as potentially being able to enhance motility in human spermatozoa.

The Flexstation assay is a HTS assay developed using a Flexstation microplate reader to measure progesterone evoked increases in intracellular Ca^{2+} in motile human spermatozoa (Martins da Silva et al., 2012). Optimised conditions were then selected to successfully screen a Chemogenomics library from the UoD DDU, The Chemogenomic library examined Tocris compounds that were selected on the supposition they would be selective and potent ion channel compounds, based on evidence from literature. This library comprised of 246 compounds with progesterone being used as an internal control for the assay. This screen identified 27 active compounds that were capable of generating a peak Ca^{2+} response after exposure to prepared spermatozoa, including compounds which are known to modulate cellular Ca^{2+}. 6 of these compounds were subjected to in-depth spermatozoa motility assessments to try and identify novel compounds that have the ability to enhance sperm motility in an *in vitro* environment (Table 5.1). 5 of these compounds will be discussed in detail in this chapter with the 6th

compound Trequinsin hydrochloride being discussed in chapter 6. The benefits of the Chemogenomics screen for a complex system such as sperm is that it allowed for the identification of a range of pharmacologically active compounds that have known specific molecular targets giving you an indication of which proteins, receptors or enzymes may be in that system. This helps to define potential targets that may be activated/ inhibited when analysing the effects of unknown compounds.

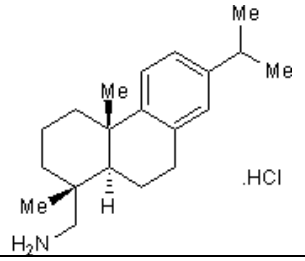
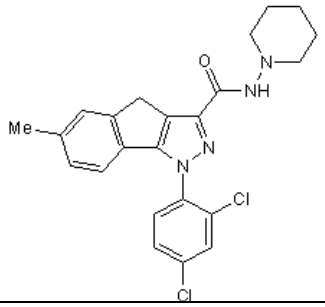
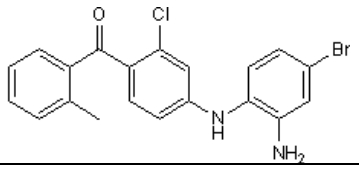
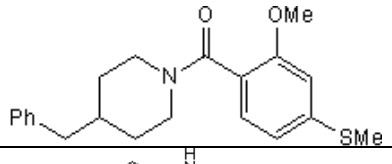
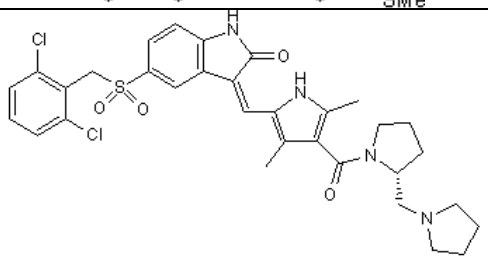
Compound name	Primary action	Chemical structure
Leelamine Hydrochloride	CB1 agonist	
GP 1a	Highly selective CB2 agonist	
EO 1428	Selective inhibitor of p38α and p38β	
JX 401	Potent, reversible p38α inhibitor	
PHA 665752	Potent and selective MET inhibitor	

Table 5.1 Summary of active compounds identified in screen of the Chemogenomics library examined for their effects on sperm motility (Biosciences, 2013a-e)

5.1.1 Cannabinoid agonists

Leelamine hydrochloride (Lylamine hydrochloride, (+)-Dehydroabietylamine) is a CB_1 receptor agonist (IC50 2.86μM) (Biosciences, 2013d) and GP1a is a CB_2 receptor agonist (Biosciences, 2013b) (Table 5.1). There are two different types of cannabinoid receptors that have been identified and cloned, both of which are members of the super-family of G-protein – coupled receptors (Howlett et al., 2002). CB_1 and CB_2 receptors are so called because they are the receptors that respond to cannabinoid drugs. The CB_1 cannabinoid receptor was first isolated in the brain and found mainly at the terminals of central and peripheral neurons but it has since been identified as being expressed in other tissues including spleen, peripheral leukocytes, uterus, testis, individual sperm cells, muscle cells and placenta (Howlett et al., 2002, Schuel et al., 2002, Rossato et al., 2005). It has been cloned from rat, mouse and human tissues and exhibits 99% homology across species (Howlett et al., 2002). The CB_2 receptor exhibits 48% homology with the CB_1 receptor and is primarily located to immune cells within and outside the central nervous system and has also been identified in human sperm cells (Ashton and Glass, 2007, Agirregoitia et al., 2010a).

Functional CB_1 and CB_2 receptors have been identified in mature human spermatozoa (Rossato et al., 2005, Agirregoitia et al., 2010a) along with mRNA and proteins for CB_1 and CB_2 receptors (Agirregoitia et al., 2010a, Amoako et al., 2013a). CB_1 has been shown to be localised to the plasma membrane of the acrosomal region, tail and midpiece of human sperm, whereas CB_2 has been found to be mainly localised in the post-acrosomal region, midpiece and tail of sperm cells (Agirregoitia et al., 2010a, Amoako et al., 2013b)

Compounds that act via the CB_1 and CB_2 receptors reproduce some of the biological actions of the cannabinoid drugs such as Δ^9-tetrahydrocannabinol (THC) which is

derived from the natural *Cannabis sativa* (Howlett et al., 2002). Along with cannabinoids, a family of unsaturated fatty acid derivatives with actions similar to cannabinoids have been identified and are known as endocannabinoids. Cannabinoids and endocannabinoids exert their effects via activation of specific cannabinoid receptors (Rossato et al., 2005, Amoako et al., 2013b). Previous studies suggest that sperm function is regulated by endocannabinoids through a dual stage-dependent effect involving both CB_1 and CB_2 receptors (Agirregoitia et al., 2010a, Maccarrone, 2008).

CB_1 and CB_2 receptors have also been associated to transduction pathways coupled to pertussis toxin-sensitive G_i/G_o proteins. Through this they negatively affect adenylate cyclase activity and production of cAMP (Howlett et al., 2002), inhibit L, N, P and Q type voltage- gated Ca^{2+} channels (Howlett et al., 2002, Howlett, 2005) and positively affect A-type and inwardly rectifying K^+ channels, phospholipase C and mitogen-activated protein kinase (Howlett, 2005, Pertwee, 1997). CB_1 receptors are also capable of coupling to G_s proteins to activate soluble adenylate cyclase (Pertwee, 1997, Glass and Felder, 1997, Calandra et al., 1999). Additionally, there have been reports that CB_1 and CB_2 receptors enhance $[Ca^{2+}]_i$ concentrations (Sugiura and Waku, 2000, Sugiura et al., 1997, Pertwee, 2005). This outlines the diversity of compounds acting on these receptors and the potential for different functional events. An understanding of this complex system is fundamental to male infertility and importantly has the possibility to provide potential targets for pharmacological intervention in male infertility (Amoako et al., 2013b).

5.1.2. Mitogen activated protein kinases

Mitogen activated protein kinases (MAPKs) are key regulatory enzymes that participate in cell growth, differentiation, survival, stress, cell cycle progression and apoptosis (Almog et al., 2008). MAPK cascades consist of levels of protein kinases that sequentially activate each other through phosphorylation. There are four major MAPK cascades known for mammals: ERK1-2, JNK 1-3, p38 α-δ and ERK5 (figure 5-1) (Gutkind, 1998, Pearson et al., 2001).

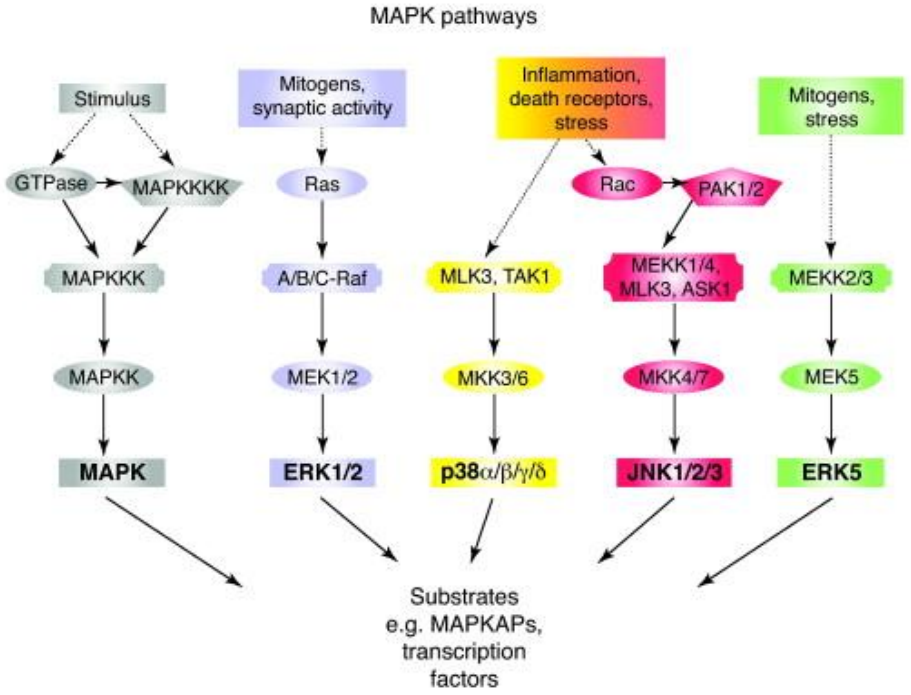


Figure 5-1. Key signalling components of the four major MAPK pathways in mammals (Berwick and Harvey, 2011)

EO1428 is a selective inhibitor of p38α and P38β_2 (IC50 0.039μM) (Biosciences, 2013a) and JX401 is a potent reversible inhibitor of p38α (IC50 32nM) (Biosciences, 2013c) (Table 5-1). p38 MAPK is now known to be a stress related activated kinase which is initiated by a stress stimuli (Almog et al., 2008). The p38 MAPK cascade consists of sequential activation of MAP3Ks, MKK3/4/6, p38 α-δ, and several mitogen activating protein kinase activating protein kinases (MAPKAPKs) (figure 5-1) (Pearson et al., 2001, Almog et al., 2008).

During spermatogenesis MAPKs function to mediate cell division, differentiation, survival and death (Wong and Yan Cheng, 2005). The knowledge of the function of MAPK in motility, capacitation and the acrosome reaction in mature spermatozoan is limited and controversial (Weidinger et al., 2005). The role of p38 was unknown until Almog *et al* identified that p38 MAPK is not only expressed in mature spermatozoa but is primarily localised to the tail (Almog et al., 2008). Almog *et al* also discovered that ERK stimulated progressive and hyperactivated motility whereas p38 MAPK acted to inhibit motility (Almog et al., 2008) (figure 5-2). Both ERK 1/2 and p38 MAPK have also been identified, positively, in having a role in PKC-mediated acrosome reaction (Almog et al., 2008).

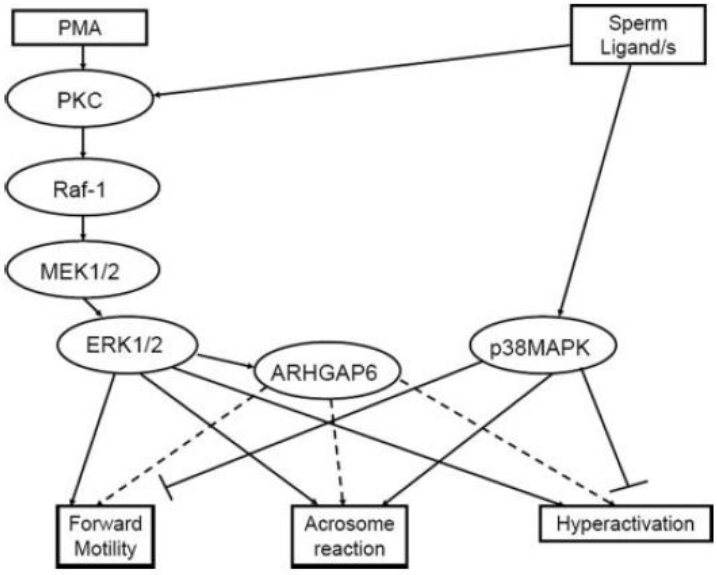


Figure 5-2 Proposed model for p38 and ERK1/2 signalling in human sperm. p38 MAPK and ERK1/2 have been identified in the tail of mature human sperm. ERK stimulates and p38 inhibits progressive and hyperactivated motility. Both p38MAPK and ERK1/2 are positively associated with the acrosome reaction. (Almog et al., 2008)

p38 MAPK is encoded by four genes that express nine isoforms with the main isoform found to be expressed in mature ejaculated human spermatozoa being p38α (Pearson et al., 2001, Almog et al., 2008). As mentioned previously both JX401 and EO1428 are selective inhibitors for p38α suggesting that these compounds should act to inhibit the p38 inhibition on sperm motility leading to increased progressive and hyperactivated

motility. p38 inhibitors have been used previously with positive effects on both progressive motility and hyperactivation (Almog et al., 2008).

5.1.3 c-MET kinase inhibitor

PHA665752 is a potent, selective 2nd generation ATP-competitive inhibitor of c-MET kinase, a receptor tyrosine kinase (Table 5-1) (IC50 9nM) (Biosciences, 2013e).

Tyrosine kinases are themselves receptors for some growth factors that regulate cell proliferation and differentiation (Ullrich and Schlessinger, 1990, Herness and Naz, 1999). The c-met proto-oncogene encodes a transmembrane glycoprotein $p190^{MET}$ tyrosine kinase receptor (Herness and Naz, 1999). The ligand for the c-met receptor has been identified and assigned as a scatter factor/ hepatocyte growth factor (SF/HGF) (Bottaro et al., 1991). SF/HGF has been identified as having potent motogenic, mitogenic and morphogenetic actions on epithelial cells and proteins have been identified in human amniotic fluid, human placental tissue, stromal components of human testis and in the ovary (Strain, 1993, Herness and Naz, 1999, Naldini et al., 1991, Lail-Trecker et al., 1998). Depuydt *et al* identified that the c-met receptor is localised in the human seminiferous epithelium and on mature spermatozoa (Depuydt et al., 1996). This same group have also shown that HGF is present in seminal plasma (Depuydt et al., 1997) and has identified relationships between the concentration of HGF in human seminal plasma and andrological diseases, including asthenozoospermia, suggesting a link between HGF and motility (Depuydt et al., 1998). SF/HGF has been found to be differentially expressed in segments of the male genital tract, highest at the cauda and distal corpus of the epididymis, in mice (Naz et al., 1994). Herness *et al* identified that the c-met receptor is present in human mature sperm cells predominantly in the acrosomal subcellular site (Herness and Naz, 1999). The c-met receptor is tyrosine phosphorylated/ autophosphorylated during capacitation through cross-talk

with other molecules (Naz, 1996) (figure 5-3). Protein tyrosine phosphorylation has a role in a variety of cellular functions including regulation of various receptors (Ullrich and Schlessinger, 1990). Tyrosine phosphorylation increases in human spermatozoa during capacitation and is an important regulatory pathway in events associated with capacitation. The signalling pathway of c-met kinases function on human sperm cells has not been elucidated but previous reports indicate that c-met and its ligand (SF/HGF) may have an important function in sperm motility owing to its motogenic properties (Naz et al., 1991, Naz, 1996, Herness and Naz, 1999, Naz and Rajesh, 2004).

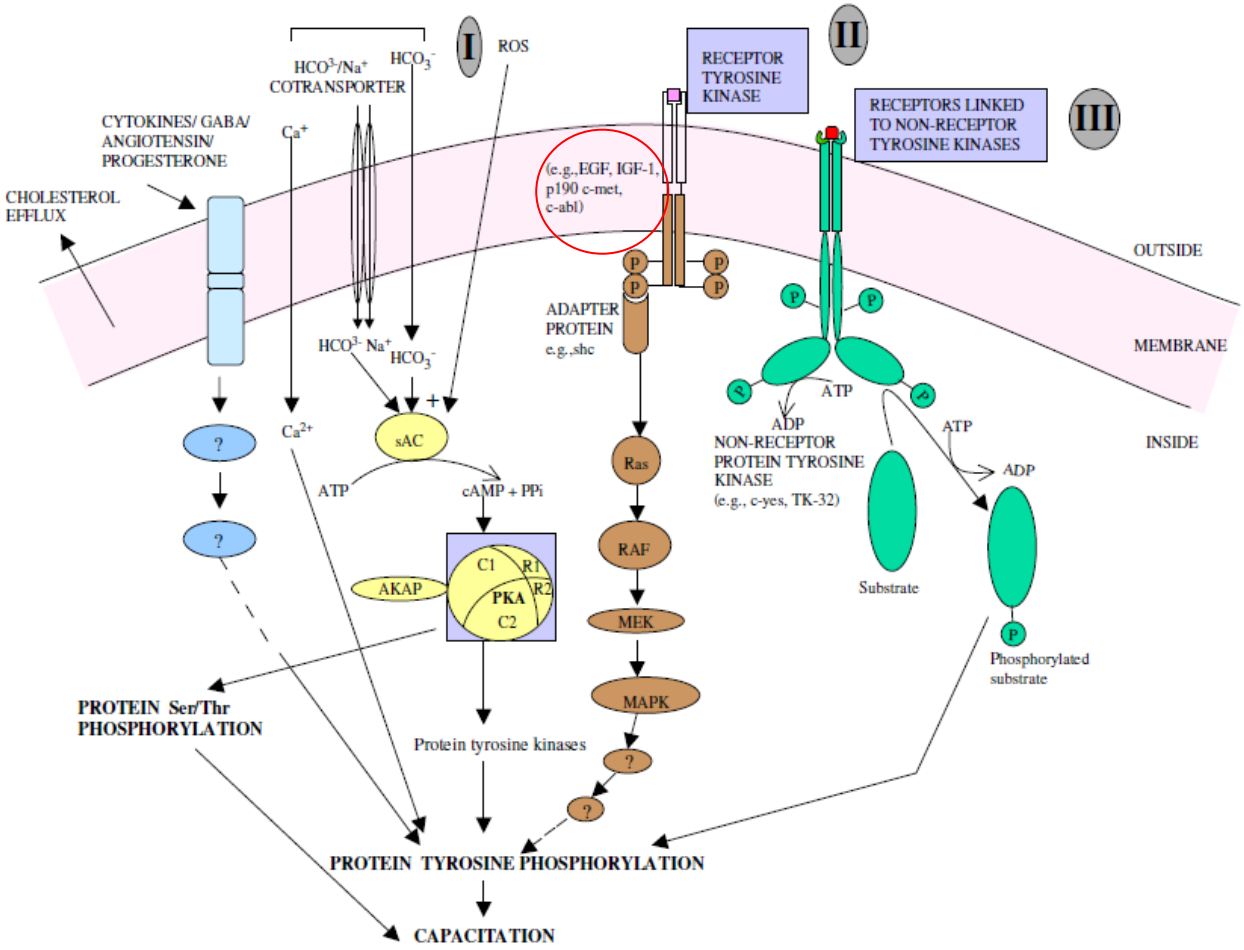


Figure 5-3. Heuristic model by Naz et al showing tyrosine phosphorylation signalling pathways in human sperm cells involved in capacitation. There are three major pathways in the sperm cell; cAMP/PKA-dependent pathway (pathway I), receptor tyrosine kinase pathway (pathway II), and non-receptor protein tyrosine kinase pathway (pathway III). The pathway by which c-met kinases function is pathway II. These cascades are not mutually exclusive and it is possible they include cross-talk amongst several molecules. Many key molecules and receptors still need to be elucidated to fully understand the molecular mechanism and signal transduction cascade involved in capacitation. (Naz and Rajesh, 2004)

5.2 Experimental Design

Semen samples obtained from donors were prepared using DGC. Each sample was diluted in either NCM or CM as appropriate to a final concentration of 20 million/ml. Samples in NCM were placed in an incubator at 37^{o}C for 15 min whereas samples in CM were placed in a 5% CO_2 incubator at 37^{o}C for 2 hrs 30 min. 99µl of semen sample was placed in a round bottom tube followed by the addition of 1µl of the appropriate compound. 1% DMSO was added to the control tube to act as a vehicle control. After re-suspending cells, 3µl of sperm suspension was loaded onto a preheated fixed chamber slide and CASA analysis conducted at 30 min intervals over a period of 3 hrs. I will only be discussing total motility, progressive motility and hyperactivation in detail. The results for VCL and ALH are available in the appendix.

5.3 Results

5.3.1 Motility assessment by CASA of five hit compounds

5.3.1.1 *Leelamine Hydrochloride*

When spermatozoa were treated with Leelamine Hydrochloride (40µM), there was a decrease in total motility, progressive motility and hyperactivation when both the 40% and 80% fraction were assessed under non-capacitating conditions (appendix). Treated cells, after capacitation, from the 40% fraction showed significant changes instantly in the percentage of motile cells ($P \leq 0.05$; figure 5-4A), whereas significant alterations in progressive motility were not observed until after 150 min ($P \leq 0.05$; figure 5-4B). Hyperactivation also decreased although this result was not significant (appendix). Results from the 80% fraction identified no significant differences between control and treated cells. However, a time- dependent decrease in hyperactivation was noted over the 3hr time course (figure 5-4C). The predominant effect exhibited by spermatozoa after prolonged exposure to this compound, in both the 40% and 80% fractions, under capacitating and non-capacitating conditions, was a decrease in all motility parameters assessed.

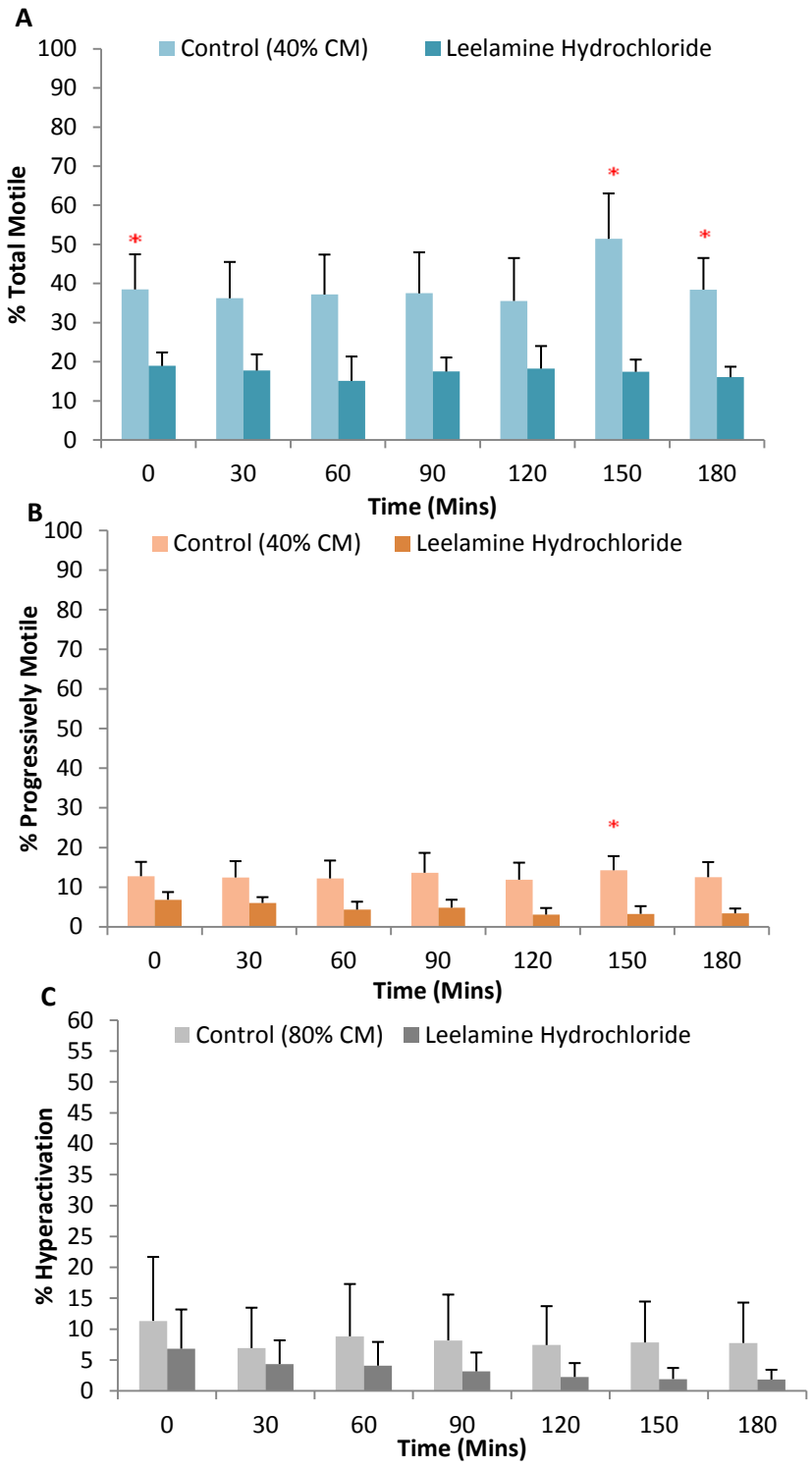


Figure 5-4. Expression of basal (control) motility values compared to Leelamine Hydrochloride treated samples capacitating conditions. (A) % total motile (40% fraction CM), **(B)** % progressively motile (40% fraction CM) and **(C)** % hyperactivation (80% fraction CM). The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min capacitating conditions comparing the difference between the control and treated samples. N= 4 for all parameters, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

5.3.1.2 *GP1a*

After evaluating the effects of a CB_1 agonist on sperm motility, GP1a, a CB_2 agonist, was examined (40μM). The results from cells treated with GP1a were similar to that of Leelamine hydrochloride with the trend exhibiting decreased motility. Once again, cells under non-capacitating conditions treated with the agonist displayed less motile cells than control in both the 40% (appendix) and 80% fractions ($P \leq 0.05$ figure 5-5A). However, progressive motility and hyperactivation were not significantly changed from control (appendix). The most substantial changes were exhibited when the sperm cells had been exposed to GP1a after capacitation. In the 40% fraction total motile, progressively motile and hyperactivation, were all significantly decreased (figure 5-5B,C&D). Cells from the 80% fraction revealed time-dependent decreases in hyperactivation ($P \leq 0.05$) after 30 min treatment (figure 5-5F). However, significant decreases in total motility were not observed until after 180 min treatment (figure 5-5E) with no changes in progressive motility observed (appendix).

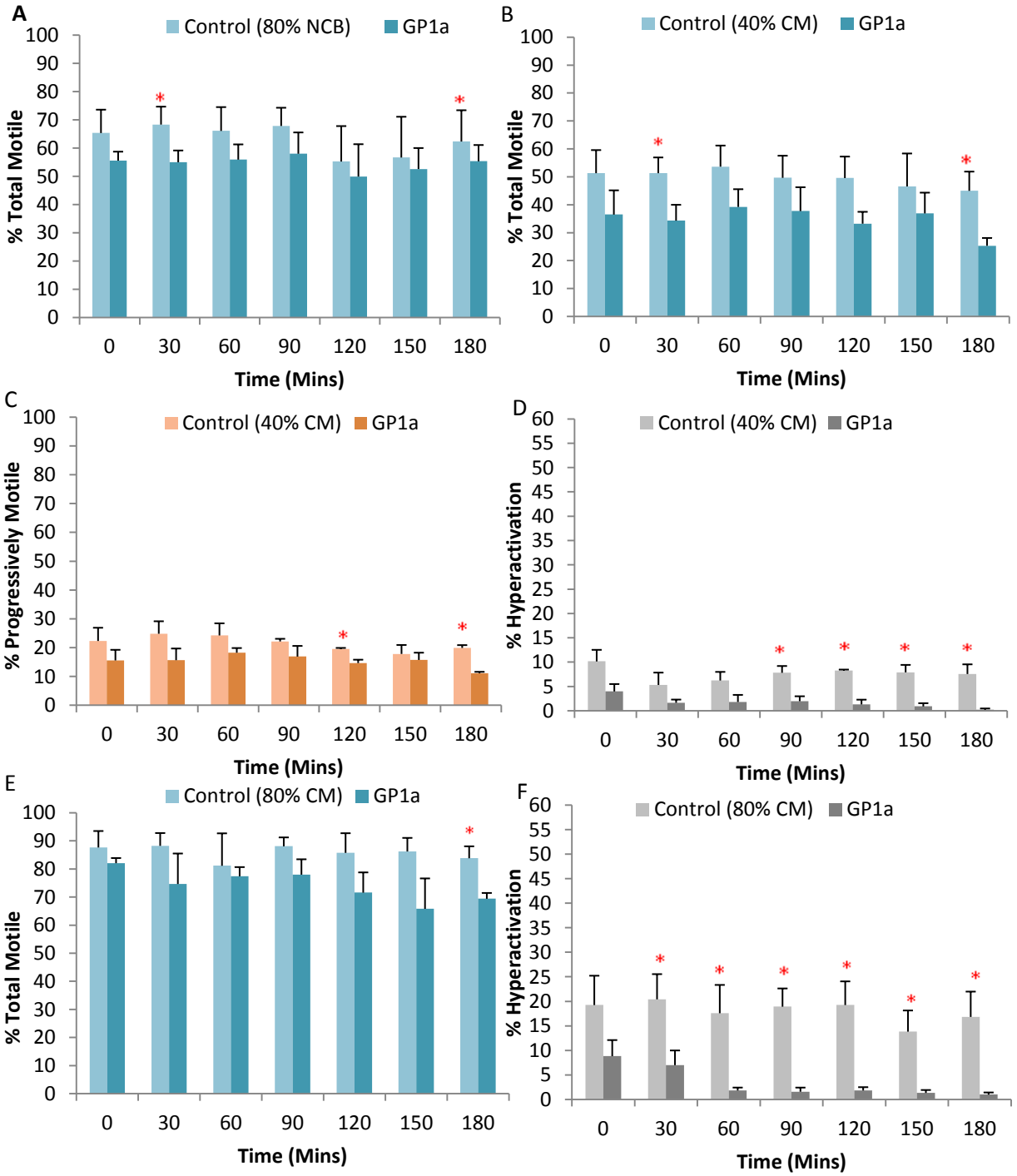


Figure 5-5. Expression of basal (control) motility values compared to treated samples in non capacitating (NCM) and capacitating (CM) media, (A) % total motile (80% fraction NCM), **(B)** % total motile (40% fraction CM), **(C)** % progressively motile (40% fraction CM), **(D)** % hyperactivation (40% fraction CM), **(E)** % total motile (80% fraction CM) and **(F)** % hyperactivation (80% fraction CM)**.** The result shown is the mean ± SE for all parameters. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

5.3.1.3 *EO1428*

CASA analysis of sperm treated with EO1428 (40μM) under non-capacitated conditions showed significant decreases in total motility and progressive motility ($P \leq 0.05$, appendix) for cells from the 40% fraction. Treated samples from the 80% fraction displayed significant decreases in total motility ($P \leq 0.05$, figure 5-6A). The percentage of progressively motile cells was observed to decrease significantly after 30 min treatment and this decrease was sustained for the remainder of the time-course ($P \leq 0.05$, figure 5-6B). However, no change was observed in hyperactivation compared to control (appendix). Cells treated with this agonist, similarly to GP1a, exhibited a higher proportion of significant decreases in sperm motility in capacitated cells compared to non-capacitated. The 40% fraction under these conditions showed significant decreases in total motility, progressive motility and hyperactivation ($P \leq 0.05$, figure 5-6 C, D and appendix). Spermatozoa from the 80% fraction displayed significant reductions ($P \leq 0.05$) in hyperactivation after 60 min an effect that was sustained over the remainder of the time-course (figure 5-6E). Kruskall Wallace statistical analysis indicated that hyperactivation was significantly lower in the treated spermatozoa ($p=0.036$) over the 3hr time-course (figure 5-6E). Progressive motility appeared to increase compared to control after 60 and 90 min treatment however, this was not a significant increase and was not sustained (appendix). Total motility remained unchanged from control (appendix).

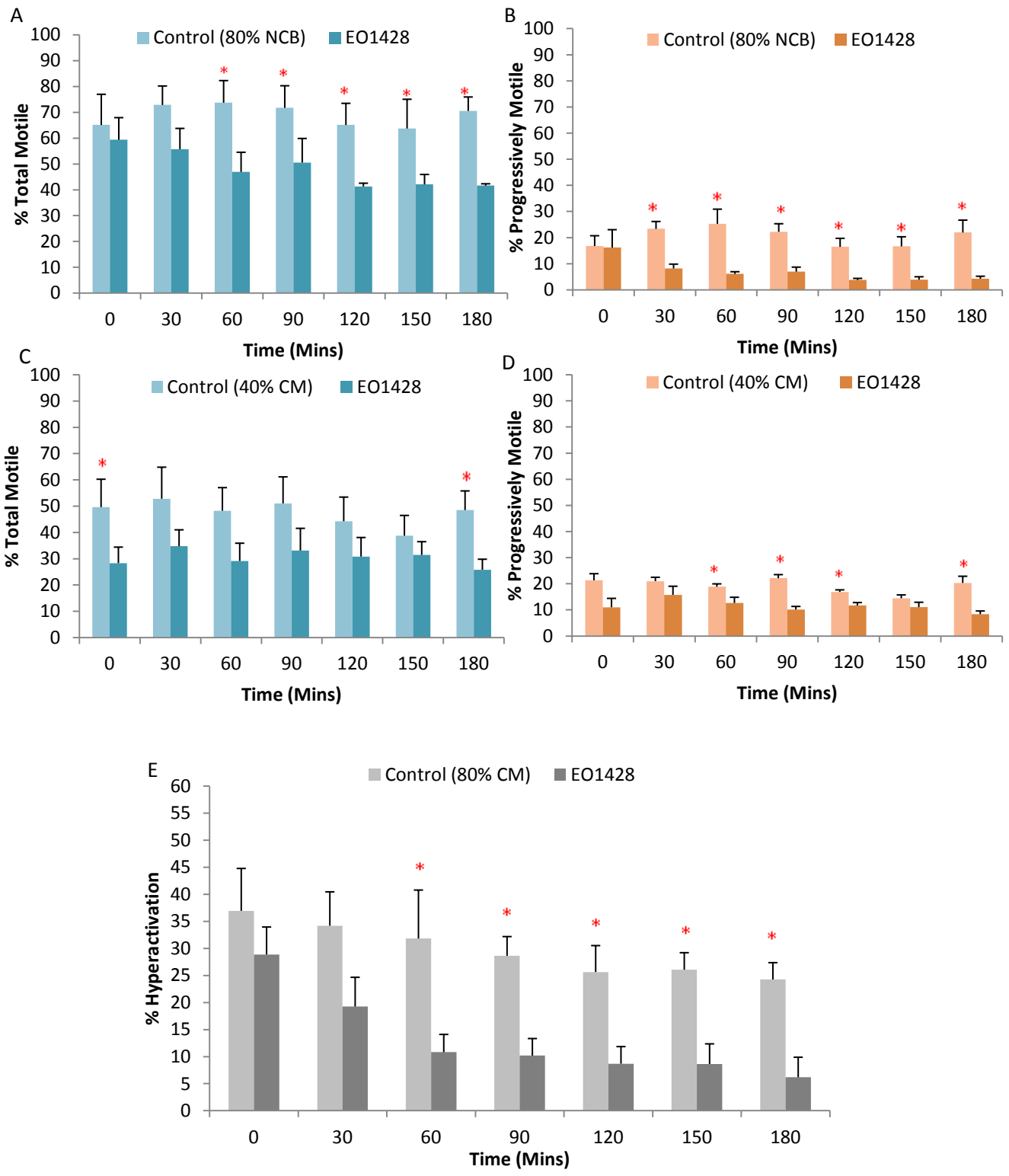


Figure 5-6. Expression of basal (control) motility values compared to EO1428 treated samples in non-capacitating (NCM) and capacitated (CM) media. (A) % total motile (80% fraction NCM), **(B)** % progressive motility (80% fraction NCM), **(C)** % total motile (40% fraction CM), **(D)** % progressive motility (40% fraction CM) and **(D)** % hyperactivation (80% fraction CM)**.** The result shown is the mean ± SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

5.3.1.4 *JX401*

In motility parameters, under non-capacitating conditions, sperm progressive motility and hyperactivation were significantly decreased ($P \leq 0.05$) for the 40% fraction (figure 5-7A & appendix respectively). No significant changes were observed in any other parameter over the time-course (appendix). For the 80% fraction both the percentage of total motile and progressively motile cells, treated with JX401 (40μM), showed significant decreases after 60 min (total motile) and 30 min (progressively motile) which is sustained for the 180min incubation (appendix and figure 5-7B). Hyperactivation was also found to be significantly lower after 30 min incubation (appendix). This compound differed to the others examined with the most significant results being produced under non-capacitating conditions instead of capacitating as shown previously. Cells from the 40% fraction, exposed to capacitating conditions, following treatment with this compound, exhibited reduced progressive motility instantly and this effect was sustained after 30, 90 and 120 min (figure 5-7C). However, no significant differences were found for any other kinematic parameter (appendix). Treated sperm collected from the 80% fraction revealed significant decreases in total motility and hyperactivation when compared to control (appendix) however, progressive motility remained unchanged from control (appendix).

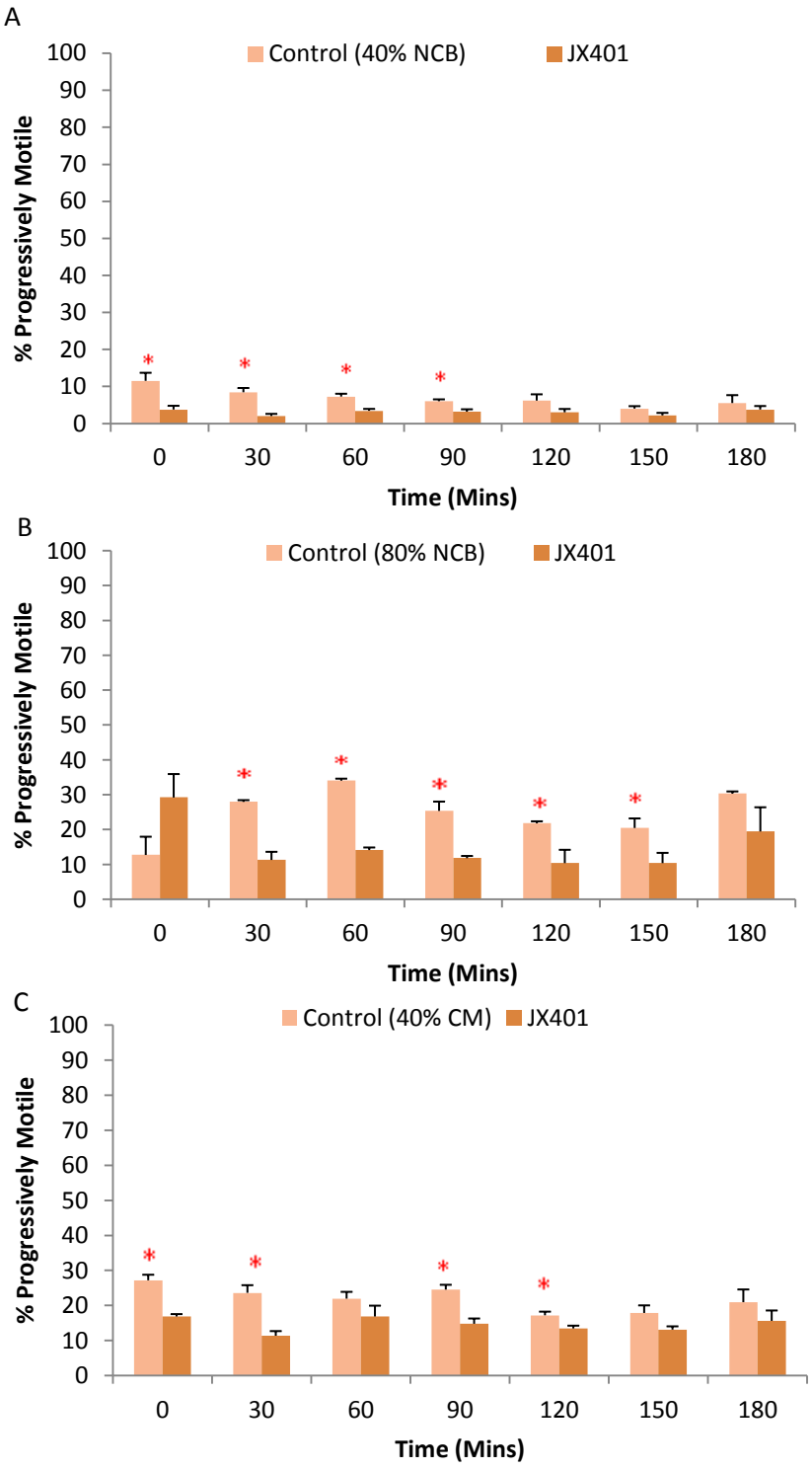


Figure 5-7. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating media. (A) % progressive motility (40% fraction, NCM), **(B)** % progressive motility (80% fraction, NCM) and **(C)** % progressively motile (40% fraction, CM). The result shown is the mean ± SE for motility parameters measured. All motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions. N= 3 for all parameters, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

5.3.1.5 *PHA665752*

PHA665752 (40µM) was the only compound that produced no significant changes from control when motility parameters were examined for the 40% fraction under non-capacitating conditions (appendix). The 80% fraction under the same (non-capacitating) conditions exhibited significant decreases ($P \leq 0.05$) in the percentage of motile cells after 90 min but was not sustained (appendix). No other parameters where significantly changed from control under these conditions (appendix). Cells that had been capacitated prior to treatment, when assessing motility, exhibited significant decreases for the percentage of total motile (180 min) and progressively motile (120 min) cells when compared to control in the 40% fraction (appendix). Similarly to GP1a and EO1428 the most significant decreases in kinematic parameters were identified from the 80% fraction after capacitation. PHA665752 was causative of decreases in hyperactivation, along with, a time-dependent decrease in the percentage of total motile cells ($P \leq 0.05$, figures 5-8A&B). Progressive motility exhibited no significant changes from control (appendix). Kruskall Wallace statistical analysis identified time-dependent significant decreases in spermatozoa treated with PHA665752 for hyperactivation (figures 5-8B).

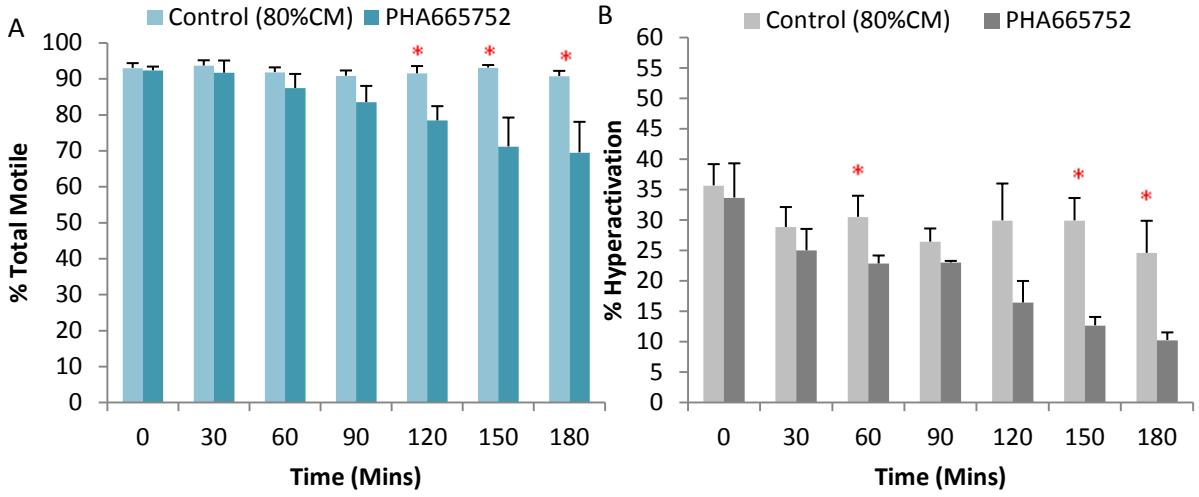


Figure 5-8. Expression of basal (control) motility values compared to treated samples in capacitating (CM) media in 80% fraction for (A) % total motile and **(B)** hyperactivation**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

5.4 Discussion

The primary aim of this chapter was to examine the effects of compounds, identified from the Flexstation high-throughput screening assay, on motility parameters of human sperm from donors and sub fertile patients. However, the predominant effect seen as a result of addition of the 5 other agonists examined (Leelamine hydrochloride, GP1a, EO1428, JX402 and PHA665752) indicated significant decreases in all motility parameters.

Cannabinoid receptor agonists increase $[Ca^{2+}]_i$ but fail to enhance sperm motility

The results of this chapter showed that motility parameters of human sperm, specifically the percentage of total motile cells, were decreased when exposed to both a CB_1 agonist; Leelamine hydrochloride, and a CB_2 agonist; GP1a. This result reflects previous studies examining the effects of cannabinoids on sperm function (Rossato et al., 2005). Endocannabinoids (a group of endogenous, lipid mediators) have been found to play a role in motility and negatively affect fertilising potential of human sperm (Francavilla et al., 2009). The two best characterised members of the endocannabinoid family are N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG, a monoacylglycerol) both of these endocannabinoids are capable of working at the type 1 (CB_1 receptor) and type 2 (CB_2 receptor) cannabinoid receptors (Howlett et al., 2002, Francavilla et al., 2009). Increases in intracellular Ca^{2+}, as a result of cannabinoid agonist addition (Howlett et al., 2002), induces production of AEA which in turn through a dual stage-dependent effect activates both CB_1 and CB_2 receptors (Marzo et al., 2005, Maccarrone, 2008, Agirregoitia et al., 2010a). The most well know endocannabinoid is *N*-arachidonoylethanolamine (anandamide, AEA) (Devane et al., 1992). The biosynthesis of AEA is complex but most of the pathways involved in the synthesis of AEA are by a specific phospholipase (NAPE-PLD) with AEA being synthesised from the precursor *N*-acylphosphatidylethanolamine (NAPE) (Okamoto et

al., 2007). The effects of AEA are terminated when AEA is taken up through an AEA membrane transporter after which it is degraded to ethanolamine ($EtNH_2$) and arachidonic acid (AA) by the fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002, Amoako et al., 2013b).

Significant levels of AEA have been found to be present in human seminal plasma, as sperm leave seminal plasma and approach the egg in the female reproductive tract; they are exposed to progressively decreasing AEA concentrations (Rossato et al., 2005, Amoako et al., 2010). It has been theorised that the high AEA concentrations observed in seminal plasma could act to maintain sperm in a quiescent metabolic state that can be activated within the female reproductive tract (Schuel et al., 2002). Once a threshold level of AEA is reached membrane hyperpolarization is triggered increasing the influx of Ca^{2+}. This theory is supported by differential expression of NAPE-PLD and FAAH that finely control levels of AEA in mouse oviduct (Wang et al., 2006). In this respect, decreasing AEA concentration in the female reproductive tract secretions could reduce its inhibitory effect on sperm, thus rendering them suitable for capacitation and gaining their fertilisation potential (Rossato et al., 2005). The existence of this AEA gradient suggests a modulatory role of the endocannabinoid system on human sperm function and could modulate sperm cell motility (Agirregoitia et al., 2010a, Amoako et al., 2013b).

Leelamine was found from the Flexstation screen to increase $[Ca^{2+}]_i$ in human sperm cells which potentially increases production of AEA leading to increased levels of AEA and a decrease in sperm function, including sperm motility, by promoting inhibition through activation of the CB_1 receptor (mechanisms described previously, figure 5-9). Similarly, the incubation of sperm cells with a selective CB_2 receptor agonist GP1a, also exhibited significant decreases in motility parameters which corroborates a previous

study by Agirregoitia *et al* who identified a shift in the proportion of cells from A grade to B grade on addition of CB_2 agonist (Agirregoitia et al., 2010a). These results suggest that cannabinoids do have a role in controlling sperm motility however it may be more beneficial for their potential therapeutic applications to further study their possible use as contraceptives rather than motility enhancers.

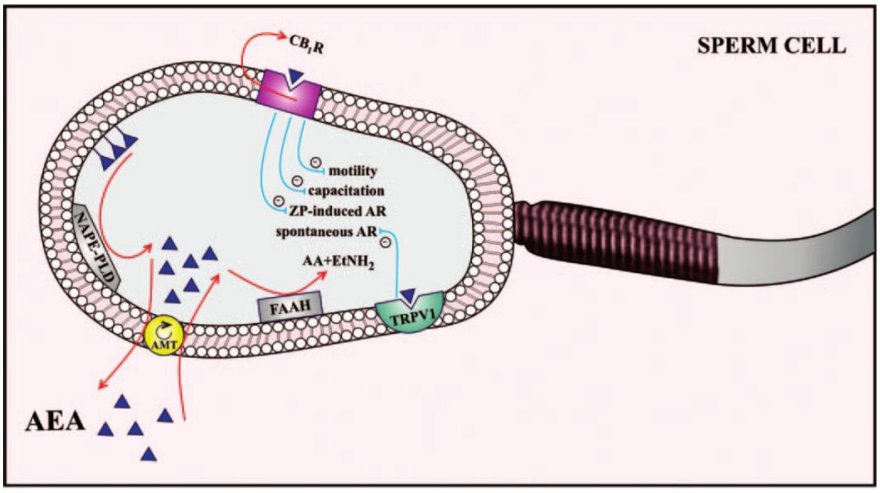


Figure 5-9. The endocannabinoid system and sperm function. Alterations in $[Ca^{2+}]_i$ prompt AEA production which binds to the extracellular site of CB_1 (CB_1R) leading to inhibition of sperm motility, acrosome reaction and capacitation. AEA is broken down via the synthesizing phospholipase NAPE-PLD and the AEA hydrolyzing FAAH which cleaves AEA into ethanolamine ($EtNH_2$) and AA. Image from Wang et al. (Wang et al., 2006)

Mitogen activated protein kinase inhibitors fail to enhance sperm motility

Treatment with both JX401 and EO1428 was found to significantly decrease total motility, progressive motility and hyperactivation of treated cells under all conditions examined. Numerous groups have published information regarding MAPK cascades involvement in spermatogenesis and spermatozoa functions, however this data is controversial and information regarding the nature of endogenous and physiologically relevant sperm- ligands, that are capable of activating MAPK's including p38, remain unknown (Weidinger et al., 2005, Wong and Yan Cheng, 2005, Almog and Naor, 2008). However, previous groups making use of p38 inhibitors in assessing sperm motility noticed increases in sperm motility and hyperactivation (Almog et al., 2008) which contradicts the results of this study. As described previously p38α has been

identified to be located at the sperm tail and has been proposed to negatively affect progressive motility and hyperactivation (figure 5-3) (Almog et al., 2008). This suggested that the use of specific p38α inhibitors would have the opposite effect allowing for improved motility and hyperactivation. As this was not seen to be the case in this study, but has been seen previously (Almog et al., 2008), it suggests the possibility that these compounds may be toxic to spermatozoa (Li et al., 2009). p38α is broadly expressed and is the most abundant p38 family member found to be present in most cell types (Porras et al., 2004). Due to the limited understanding of p38 signalling pathways in sperm it could be possible that inhibition of p38α could be detrimental not only to sperm motility but to other vital functions associated with cell survival. Almog et al proposed that p38 works in association with ERK1/2 to regulate sperm motility with ERK1/2 promoting and p38 inhibiting motility (Almog et al., 2008) however, Weidinger et al published an opposite report that concluded that ERK1/2 inhibited sperm motility (Weidinger et al., 2005). In this instance, if p38 and ERK1/2 are regulating motility, and ERK1/2 is actually inhibiting motility, then it is possible that p38 might be promoting motility and addition of p38 inhibitors would surmise that you would see a decrease in sperm motility under these circumstances. An earlier study, on demembranated fowl sperm, had suggested that the MAPK pathway had a function in sperm motility after noticing inhibited flagella motility on addition of MAPK inhibitors (Ashizawa et al., 1997). p38 is known to play a vital role in germ cell development, specifically in spermatogenesis having a role in disturbing the blood testes barrier to allow preleptolene spermatocytes to migrate to the adluminal compartment (Almog and Naor, 2010). Previous studies on mice have also indicated the importance of p38 through targeted inactivation of the p38α gene which resulted in embryonic death due to a placental defect (Adams et al., 2000). If p38α inhibitors were proposed for use clinically in increasing sperm motility the potential negative effects on the prospective

embryo (Adams et al., 2000) would need to be considered suggesting that p38α inhibitors may not be of use in pharmacological intervention without causing systemic toxicity and embryonic death (Adams et al., 2000, Li et al., 2009).

C-met inhibitors decrease motility when spermatozoa are in a capacitated state

The effect of c-met on human ejaculated spermatozoa is still not clear with published results being controversial (Wiltshire et al., 2000, Catizone et al., 2006). C-met receptor induces its biological activities through binding its ligand; HGF/SF which has potent mitogenic, motogenic and morphogenetic activities (Herness and Naz, 1999, Kitamura et al., 2000, Wiltshire et al., 2000). Transgenic studies have shown that in mice targeted HGF gene disruption is lethal *in utero* (Uehara et al., 1995) identifying the importance of action through this ligand and its associated c-met receptor. This study suggests that inhibiton of the c-met receptor would prevent HGF binding resulting in a similar effect to that seen in the knock out study. Naz *et al* have previously examined the relationship between HGF sperm motility in murine models and their results suggested HGF may be involved in the attainment of motility in mammalian sperm (Naz et al., 1994). HGF has also been reported to have a positive effect on epididymal sperm motility in rats (Catizone et al., 2006). This same group identified c-met receptors to be localized both on the head and the flagellum of isolated epididymal spermatozoa and suggested in rats that HGF/c-met are related to epididymal acquisition and maintenance of sperm motility (Catizone et al., 2006). This data contrasts with studies on human sperm where it has been reported that HGF does not significantly maintain or alter human sperm motility (Kitamura et al., 2000, Wiltshire et al., 2000). This conflicting data suggests that the effects of HGF/c-met function differently in rodents than in humans (Catizone et al., 2006). The data available from studies on human ejaculated spermatozoa report that HGF has no active role in controlling sperm motility however both studies suggest the possibility of HGF playing a role in epididymal maturation and maintenance of motility

(Kitamura et al., 2000, Wiltshire et al., 2000). The results of this study corroborate that of Wiltshire *et al* and Kitamura *et al* suggesting that HGF/c-met do not appear to control ejaculated human sperm motility. Use of PHA665752 (c-met inhibitor) when in combination with spermatozoa under non-capacitating conditions exhibited no changes to motility parameters compared to control. This inhibition of c-met would have no effect on sperm motility if HGF/c-met are not associated with controlling motility. However, these results are not conclusive and under capacitating conditions the percentage of motile, progressively motile and hyperactivated cells displayed time-dependent decreases which could support the role for c-met in maintaining motility with the use of inhibitors preventing this action. Further evidence supporting that HGF may have a supporting role in maintaining sperm motility is that the c-met receptor has been found to be tyrosine phosphorylated/ autophosphorylated during capacitation, an effect that is enhanced by HGF/SF (Herness and Naz, 1999). Tyrosine phosphorylation of sperm membrane proteins is vital in development of fertilising capacity, the c-met receptor may have involvement in this process through its ability to be phosyphorylated/ autophosphorylated at tyrosine residues (Herness and Naz, 1999). Inhibition of c-met would inhibit tyrosine kniase activity decreasing hyperactivation (Bajpai and Doncel, 2003) a result which is corroborated by this study. Tyrosine phosphorylation is a hallmark of capacitation in mammalian sperm (Visconti et al., 1995b, Signorelli et al., 2012) and leads to increases $[Ca^{2+}]_i$ and activation of Catsper channels (Wennemuth et al., 2003, Signorelli et al., 2012) suggesting that c-met could play a role in increasing sperm motility. Inhibition of c-met would therefore decrease motility in capacitated sperm as seen is this study. The results of this study and previous publications suggest that C-met inhibitors would not be beneficial clinical treatments for sperm motility (Depuydt et al., 1998, Herness and Naz, 1999, Kitamura et al., 2000, Wiltshire et al., 2000).

CHAPTER 6

Motility Analysis of the PDE inhibitor Trequinsin Hydrochloride

6.1 Introduction

6.1.1 Phosphodiesterase inhibitor

Trequinsin hydrochloride is a potent phosphodiesterase inhibitor of cGMP-inhibited phosphodiesterase (PDE3) (Biosciences, 2013f) (IC50 250pM) (Table 6-1).

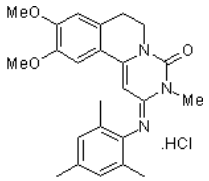
Compound name	Primary action	Chemical structure
Trequinsin Hydrochloride	Selective inhibitor of PDE3	

Table 6.1 Summary on Trequinsin Hydrochloride identified from screen of the Chemogenomics library (Biosciences, 2013f)

Cyclic adenosine monophospahate (cAMP) plays an important role in the signalling pathways that regulate sperm motility (Tash, 1990), capacitation and the acrosome reaction (Visconti et al., 1995c). Levels of cAMP are regulated by two key enzymatic reactions: (i) soluble adenylyl cyclase which produces cAMP from adenosine triphosphate (ATP) and (ii) cyclic nucleotide phosphodiesterases (PDEs). PDEs degrade cAMP to 5'AMP and are important in controlling cyclic nucleotide levels in spermatozoa (Lefievre et al., 2002). There are at least 11 identified families of phosphodiesterases, many with several different isoforms, which are classified according to kinetics, mechanism of regulation, affinity and substrate specificity (Beavo, 1995, Soderling and Beavo, 2000). PDEs are highly specific for hydroloyis of cAMP (PDE 4, 7 and 8),cyclic guanine monophosphate (cGMP) (PDE 5, 6 and 9) or they hydrolize both cAMP and cGMP (PDE 1, 2, 3, 10 and 11) (Lefievre et al., 2002). Lefievre *et al* (2000) identified that the PDEs present in spermatozoa have a higher affinity for cAMP after observing that the rate of hydrolysis of cAMP in sperm by PDEs is 3 fold higher than that of cGMP (Lefievre et al., 2000). Currently PDE 1 (Vasta et al., 2005), PDE 2, PDE 3, PDE 4 and PDE 5, PDE 8 (Richter et al., 1999) and PDE 11 (Yuasa et al., 2001) have been identified in mammalian and human testis and spermatozoa along with mRNA transcripts being identified for six types of PDEs (Fisch

et al., 1998, Lefievre et al., 2000, Lefievre et al., 2002, Richter et al., 1999, Yan et al., 2001, Fisher et al., 1998, Fawcett et al., 2000, Coskran et al., 2006).

As mentioned, PDE3 has mixed specificities with cGMP acting to inhibit the hydrolysis of cAMP by PDE3 (Lefievre et al., 2002). The activity of PDE3 is regulated by feedback mechanisms involving cAMP dependent PKA phosphorylation (Visconti et al., 1997) (figure 6-1).

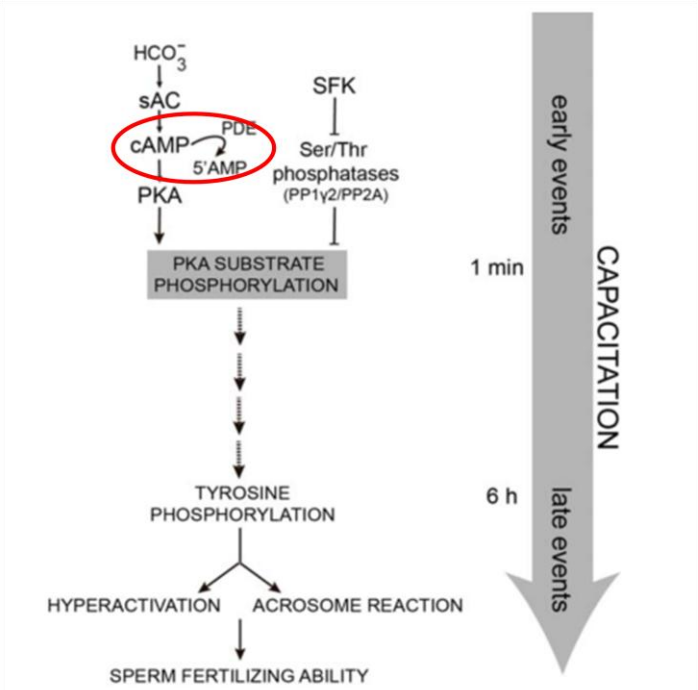


Figure 6-1.Signaling pathways involved in sperm capacitation. Two pathways involved in human sperm capacitation, one corresponds to the activation of PKA by cAMP, and the other involves downregulation of Ser/Thr phosphatases by the Src family kinase (SFK). Both pathways lead to PKA substrate phosphorylation followed by tyrosine phosphorylation resulting in capacitation. PDEs play a role in the pathway by degrading cAMP to 5'AMP (red circle). (Battistone et al., 2013)

cAMP is a key second messenger in protein tyrosine phosphorylation, motility and capacitation (Tash, 1990). Previous studies investigating the effects of PDE3 specific inhibitors have identified that they act by increasing the level of cAMP in sperm (Lefievre et al., 2002). This, combined with the discovery that Trequinsin hydrochloride increases levels of intracellular Ca^{2+}, led to the investigation of the effect of Trequinsin hydrochloride on sperm motility.

6.2 Experimental Procedure

Experimental procedure for sperm preparation and CASA analysis is identical to that as described for compounds in chapter 5.

Kremer mucus penetration tests were conducted using methylcellulose prepared in NCM. Capillary tubes were placed in the methylcellulose for 30 min at 37^{o}C allowing the tubes to fill. One end was then sealed and the open end was placed in prepared sperm (donor and patient samples, see chapter 2 for details). Sperm preparations and capillary tubes were incubated in appropriate incubators (CO_2 or normal) for 1 hr. The capillary tubes were then removed and the open ends sealed with plasticine. The tubes were marked at 1 cm and 2 cm points before being viewed on a microscope using a 20X objective. Results were normalised to parallel untreated 1% DMSO controls.

Assessment of the acrosome reaction was conducted by staining with PSA labelled FITC. Sperm were prepared through DGC, capacitated for 2 hrs 30 min before treatment with the appropriate drug for 1hr. CM only was used as a negative control (1 hr), 1% DMSO was used as a vehicle control (1 hr) and Ca^{2+} ionophore was used as a positive control (15 min). Sperm were washed twice and re-suspended in sodium chloride before being smeared on a microscope slide. After air drying the smear was fixed in 95% (v/v) ethanol before the addition of PSA labelled FITC. After 1 hr the slides were washed three times with PBS and mounted. Analysis was conducted using the fluorescence setting on an EVOS xl digital inverted microscope.

The hypo-osmotic swelling test was used to examine sperm vitality after drug treatment. Sperm suspensions were further incubated with hypo-osmotic medium and sodium citrate dihydrate at 37^{o}C in 5% CO_2 for 30 min. Sperm with coiled tails were counted as viable.

For further details refer to chapter 2.

6.3 Results

6.3.1 CASA motility analysis of Trequinsin Hydrochloride

It was clear that sperm samples under non-capacitating conditions that were then exposed to Trequinsin exhibited the most dramatic responses from both the 40% and 80% fraction. Progressive motility was significantly increased instantaneously after treatment with the compound (P=0.002) and this increase was sustained for the entire time-course ($P \leq 0.05$) (figure 6-2A). The greatest increment in hyperactivation, when stimulated by Trequinsin, was found in the 40% fraction under non-capacitating conditions after 90 min ($P \leq 0.05$) which continued to be significantly increased for the remainder of the time-course ($P < 0.005$) (figure 6-2B). However, total motility remained unchanged from control (appendix).

Spermatozoa from the 80% fraction (high quality sperm) exhibited a pronounced increase in progressive motility when treated with Trequinsin, after incubation at 37^{o}C, in non-capacitating conditions. This statistical increase in progressive motility was sustained for the entirety of the 3 hr time-course (0-60 min, 150 & 180 min $P \leq 0.05$, 90 & 120 min $P < 0.005$*)* (figure 6-2C). Similarly to the 40% fraction the percentage of hyperactivated cells in the 80% fraction was significantly higher than control after 90 min incubation with the compound *(*$P \leq 0.05$, figure 6-2D). Furthermore, hyperactivated motility was increased after 150 min and 180 min treatment ($P < 0.005$ & $P \leq 0.05$ respectively). This increase in progressive motility and hyperactivation supports the knowledge that PDE inhibitors increase cAMP leading to capacitation (Lefievre et al., 2000, Lefievre et al., 2002). However, similarly to the 40% fraction there was no significant change in total motility for this parameter (appendix). When sperm cells were exposed to Trequinsin after capacitation the resulting effect on motility was the opposite of non- capacitating conditions. The 40% fraction exhibited significant decreases in motility after 180 min incubation with a steady decline evident over the

entire time-course for the percentage of total motile, progressively motile and hyperactivated cells (appendix). Similarly, to the 40% fraction, the 80% fraction demonstrated a noticeable decline in motility which was most evident and significant in the percentage of hyperactivated cells after 150 and 180 min treatment (P=0.041 & P=0.004 respectively) (appendix and figure 6-2E). No change was observed for both the 40% and 80% fractions after capacitation for the percentage of total motile and progressively motile cells (appendix).

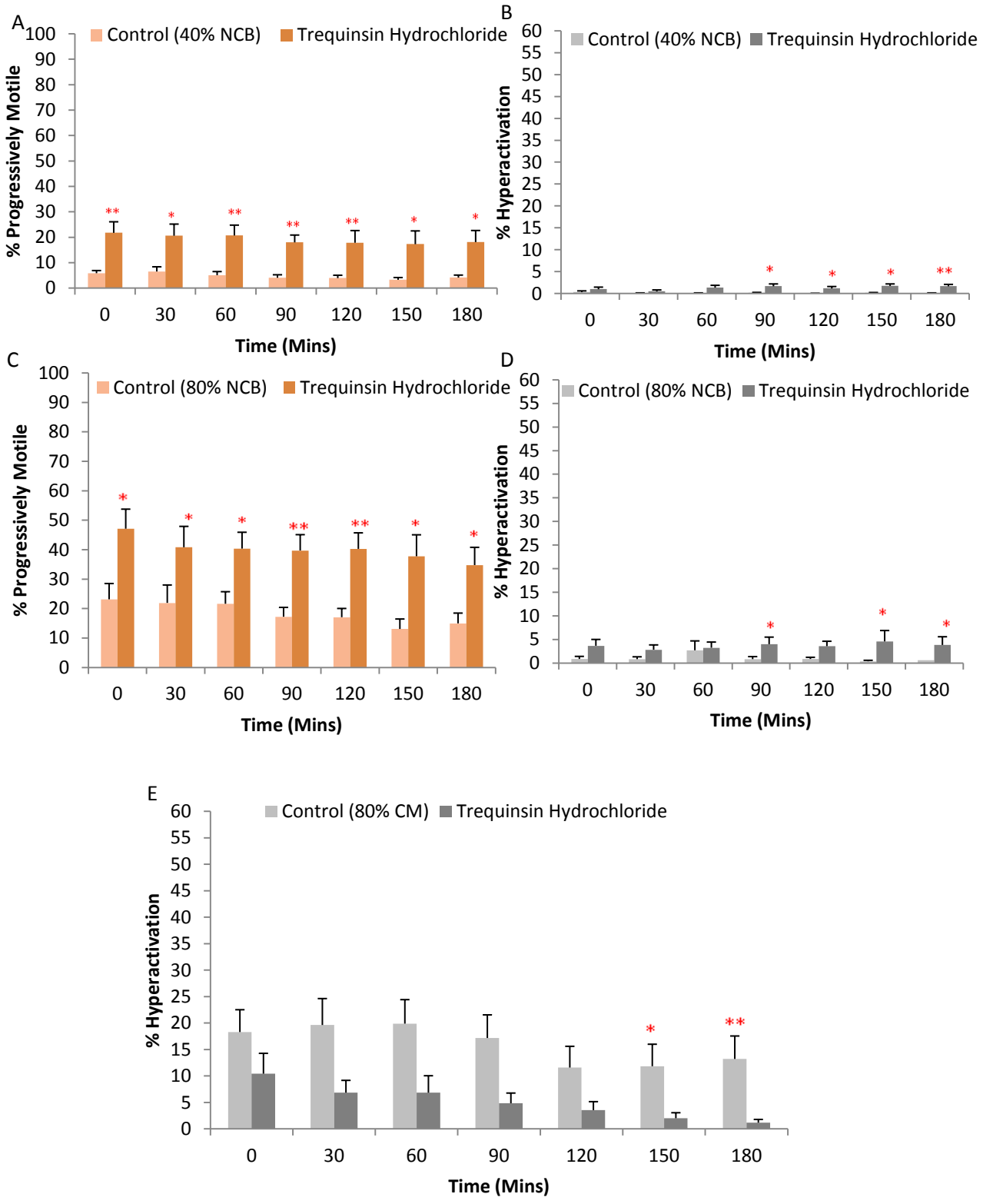


Figure 6-2. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) and capacitating (CM) media for (A) % progressively motile (40% fraction NCM), **(B)** hyperactivation (40% fraction NCM), **(C)** % progressively motile (80% fraction NCM), **(D)** hyperactivation (80% fraction NCM) and **(E)** hyperactivation (80% fraction CM)**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 6 for both parameters, * indicates a significant increase ($P \leq 0.05$), ** indicates $P \leq 0.005$ between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

The dramatic opposing effects seen in response to treatment with Trequinsin under non-capacitating and capacitating conditions directed analysis to compare the Trequinsin stimulated increase, in both the 40% and 80% fraction, under non-capacitating conditions, to that of the controls after capacitation (figure 6-3). When comparing the motility parameters from sperm cells, in the 40% fraction, no significant difference was noted between capacitated control and the treated non-capacitated sperm cells suggesting that the treated non-capacitated sperm are now acting similarly to a capacitated sample (figure 6-3A&B). Treatment with Trequinsin increased progressive motility in the treated, non-capacitated cells to within the range of control capacitated cells on instant addition of the agonist. These parameters remained similar to the capacitated control throughout the time-course until it reached 180min incubation when it was found that all parameters assessed exhibited a significant decrease compared to capacitated control ($P \leq 0.05$, figure 6-3A&B and appendix). The percentages of total motile cells were also increased in the treated, non-capacitated sample to the same as the capacitated control instantaneously although, at the 90min and 180min time-points the percentage of total motile cells was significantly decreased in the treated samples ($P \leq 0.05$ and $P \leq 0.005$ figure 6.-3A). This could indicate that the action of the compound may not last past the 3hr time course and would need testing for a longer period of time to examine any lasting motility alterations.

The same comparison was run for the 80% capacitated control and the 80% non-capacitated treated sample. The similarities in the 80% fractions were not as great as those noted for the 40% fraction. After 0 min the only parameter that was similar to the capacitated control was progressive motility. The percentage of total motile cells were similar to the capacitated control after 30 min incubation and remained this way until the 3 hr time point when it was found to have significantly decreased ($P=0.41$, figure 6-

3C). The percentage of progressively motile cells after 30 min treatment was observed to be similar to the capacitated control however; hyperactivation was significantly higher in the capacitated control at this point ($P \leq 0.05$, appendix). Hyperactivated motility displayed variable results that were only similar to the capacitated control after 90, 150 and 180 min (appendix). The percentage of progressively motile cells, after treatment with Trequinsin, increased instantaneously, an effect that was sustained with the exception of 90 and 180 min incubations (figure 6-3D).

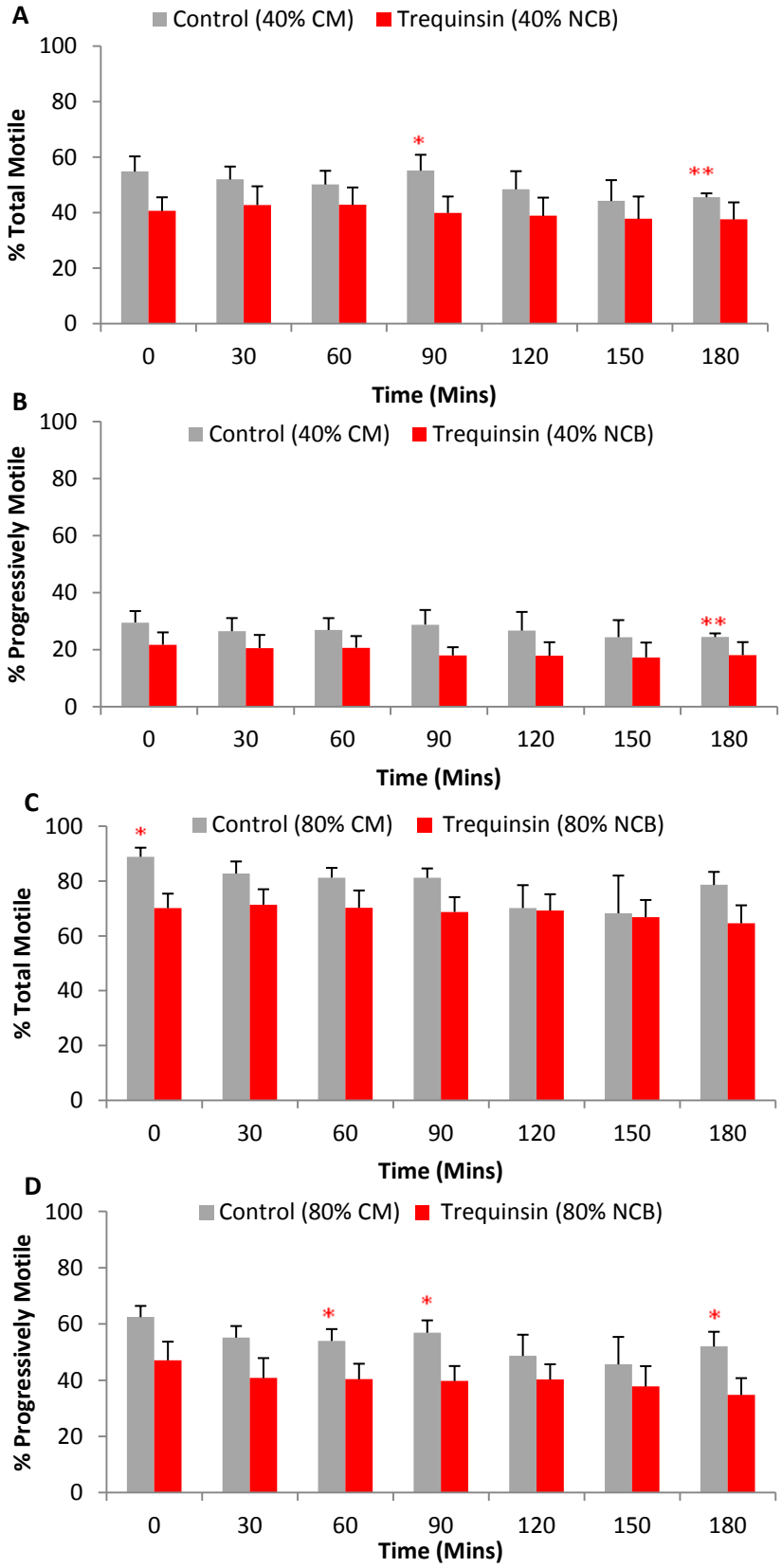


Figure 6-3. Comparing basal (control) motility values under capacitating conditions to Trequinsin treated non-capacitated samples. (A) % total motile (40% fraction), **(B)** % progressively motile (40% fraction), **(C)** % total motile (80% fraction) and **(D)** % progressively motile (80% fraction)**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min. N= 6 for all parameters, * indicates a significant increase ($P \leq 0.05$), ** indicates $P \leq 0.005$ between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskall-Wallace test).

6.3.2 Examination of sperm motility through an artificial viscous medium: methylcellulose (Kremer mucus penetration test)

To examine the ability of sperm treated with Trequinsin Hydrochloride to penetrate cervical mucus, a viscous medium methylcellulose, was used as a cervical mucus substitute in the Kremer mucus penetration assay. Previous studies have reported that sperm penetration into mucus correlates with their functional capacity and fertilisation rates both *in vivo* and *in vitro* (Aitken et al., 1992, Barratt et al., 1989, Eggert-Kruse et al., 1989). Sperm cells were incubated with Trequinsin for 1hr before cell penetration was analysed.

The initial Kremer penetration assay was conducted on capacitated sperm from the 80% fraction using progesterone as a positive control. Progesterone has previously been shown to increase sperm penetration under these conditions (Alasmari, 2013) and was used as a positive control in the Flexstation assay screen. Capacitated cells from the 80% fraction, incubated with Trequinsin, failed to increase penetration into the viscous medium. However, under the same conditions 3.6µM progesterone stimulated significant increases in cell penetration at both 1 cm and 2 cm ($P \leq 0.005$, figure 6-4A).

CASA analysis identified significant increases in motility in the 40% fraction under non-capacitating conditions although, this was not reflected in 80% capacitated samples. To examine whether this effect was the same in this functional motility assay analysis was conducted on cells from the 40% fraction under non-capacitating conditions. Under these conditions Trequinsin treated cells significantly increased penetration at both distances ($P \leq 0.005$, figure 6-4B). This result corroborates the CASA analysis indicating that Trequinsin is capable of increasing motility in non-capacitated cells. Treatment with progesterone under these conditions did not significantly change the proportion of cells penetrating at 1 cm although, at 2 cm the proportion of cells was significantly increased ($P \leq 0.005$, figure 6-4B).

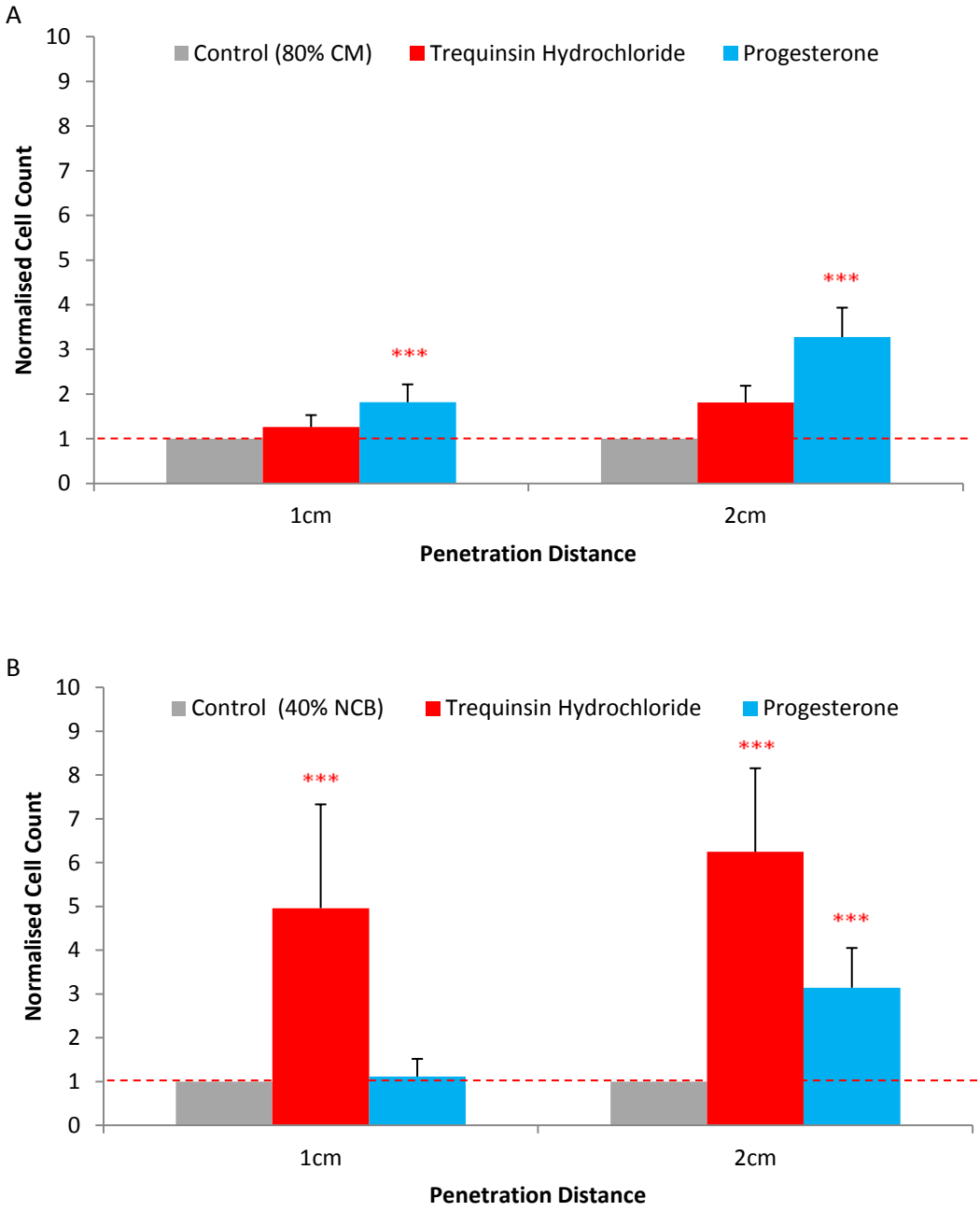


Figure 6-4. Trequinsin induced penetration of sperm cells into viscous medium. Cells from **(A)** 80% fraction under capacitating conditions and **(B)** 40% fraction under non-capacitating condition were evaluated for the number of sperm penetrating to 1 cm and 2 cm**.** The cells were counted and normalised to values in untreated controls (red dashed line). N= 5 for all experiments and all values are the mean ± SE. ***- indicates a significant difference from the control, considered to be $P \leq 0.005$ & $P \leq 0.001$ as assessed by non-parametric Mann Whitney U test.

6.3.3 Vitality analysis (HOS test) of treated sperm cells

This study utilised the hypo-osmotic swelling test in an attempt to analyse the effect seen in capacitated sperm after 3 hrs incubation with Trequinsin. Both the CASA analysis and Kremer mucus penetration test identified a decrease in sperm motility in the 80% fraction under capacitating conditions. To ensure that addition of this compound to human sperm is not toxic over long periods of time a vitality assessment was conducted.

Donor spermatozoa were capacitated for 2 hrs 30 min before being incubated with Trequinsin for a period of 3 hrs. Following incubation the vitality of the sperm cells was assessed. The result identified a mean of 92.5% of living cells with the results ranging from 88% - 99% from the 3 donor samples assessed (figure 6-5). This result was not significantly different from control suggesting that this compound is not killing the cells.

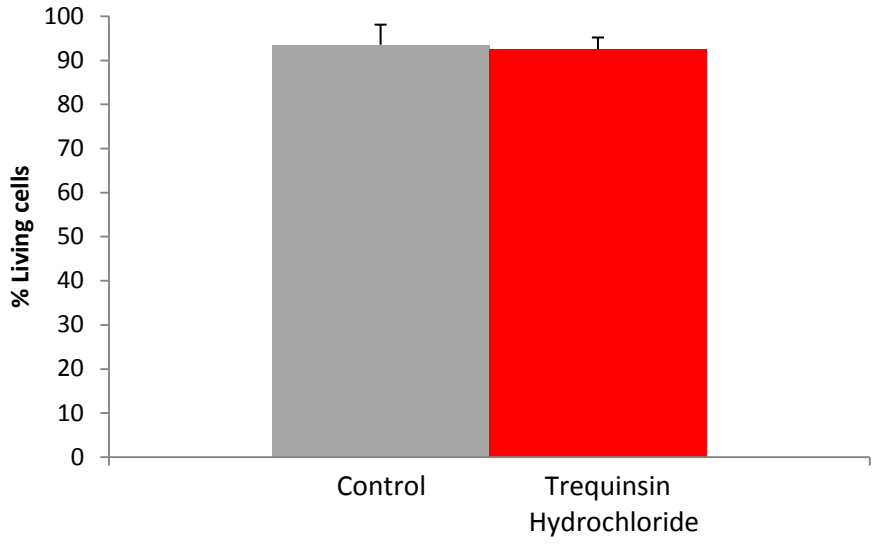


Figure 6-5. Vitality analysis after incubation with Trequinsin. Trequinsin Hydrochloride (red bar) was assessed for its effects on sperm vitality after incubation with sperm cells for a period of 3 hrs. All values are the mean ± SE, n=3 for each experiment. No statistical difference was noted from control (grey bars). Analysis was conducted using Pearson's Chi squared test and significance was assessed as $P \leq 0.05$.

6.3.4 Response of patient samples to incubation with Trequinsin Hydrochloride

To evaluate what effect Trequinsin may have in a clinical setting the same analysis were performed on treated patient samples.

6.3.4.1 *CASA Motility analysis of patient samples pre-treated with Trequinsin*

Patient samples provided by the ACU, after capacitation, were treated with Trequinsin. All samples examined were from patients attending the ACU for IVF and motility analysis was conducted over a period of 2 hrs using CASA (see chapter 2 for detailed methods). Results for each patient were analysed independently (Table 6.2). Treated patient samples exhibited significant increases in VCL (8/9) and ALH (9/9) resulting in significant increases in hyperactivated motility (Table 6-2). Following an increase in ALH, LIN was significantly decreased in 8/9 patients along with progressive motility. However, one patient did show significant increases (P8) in progressive motility but this result was highly variable for this patient across the time-course (Table 6-2). The results for all samples, regarding total motility, were variable however the predominant response showed no change in the percentage of motile cells (Table 6-2). Significant increases were observed from this CASA analysis for hyperactivation but only 2/9 patients exhibited significant increases in all the four time points, with 1 patient exhibiting significant increases after 60 min. 3/9 patients displayed no change in hyperactivation with the remaining 3 patients exhibiting variable results (Table 6-2).

Overall the result of treatment on patients with Trequinsin hydrochloride was better than that of the donor response under the same conditions (80% fraction, capacitated). Donors exhibited significant increases in VCL and hyperactivation which is opposite to results obtained for the patient samples under the same parameters (Table 6-2). There also were no significant differences noted in ALH for treated donors where there were clear increases in ALH in patient samples.

Trequinsin Hydrochloride												
	VCL				LIN				ALH			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	✓	✓	✓	✓	×	×	×	×	✓	✓	✓	✓
P2	–	–	✓	✓	–	–	–	–	✓	✓	✓	✓
P3	–	✓	✓	✓	–	×	×	×	–	✓	✓	✓
P4	–	–	–	–	×	×	×	×	✓	✓	✓	✓
P5	✓	✓	✓	✓	×	×	×	×	✓	✓	✓	✓
P6	✓	✓	–	✓	–	×	–	×	–	✓	–	✓
P7	–	–	✓	–	×	–	×	×	–	–	✓	✓
P8	✓	–	–	–	×	×	×	✓	✓	✓	✓	–
P9	✓	–	–	–	–	–	×	×	–	–	✓	✓
	Total Motile				Progressively Motile				Hyperactivation			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	–	–	×	×	×	×	×	×	✓	✓	✓	✓
P2	×	–	×	–	×	–	×	–	–	–	✓	✓
P3	×	–	–	×	–	–	–	×	–	–	✓	–
P4	–	×	×	×	×	×	×	×	–	–	–	–
P5	×	–	–	–	×	–	×	–	✓	✓	✓	✓
P6	–	–	–	–	–	×	–	–	–	–	×	–
P7	–	×	–	–	×	–	–	–	–	–	✓	–
P8	–	✓	–	✓	–	✓	×	✓	–	–	–	–
P9	–	×	×	×	–	–	×	×	–	–	–	–

Key

✓=Increase

× = Decrease

– = No change

Table 6.2 Identification of motility kinematics analysed by CASA, from patient samples, after incubation with Trequinsin Hydrochloride. Motility parameters after stimulation with Trequinsin Hydrochloride were evaluated from 9 patients undergoing IVF at Ninewells ACU. The table identifies the 6 motility parameters evaluated for each patient over a period of 2 hrs. Statistical significance (see key) was determined from comparison with control ± SD. If the agonist response was greater than or less than control it was considered as a significant result.

6.3.4.2 *Kremer mucus penetration assay to assess functional motility of patient sperm treated with Trequinsin Hydrochloride*

Donor samples exhibited significant increases in sperm penetrating ability when under non-capacitating conditions. When evaluating capacitated cells an increase compared to control was observed however it was not significant. The patient samples available for analysis were all IVF so had been capacitated for treatment. Although, we have previously shown that Trequinsin does not seem to influence capacitated cells as much as non-capacitated it was necessary to use these patient samples. The result from this assay confirmed the results observed from donor samples with no change compared to control at the 1 cm distance and a slight increase at the 2 cm distance (figure 6-6). However, this increase similarly to donors did not reach a significant level. Progesterone once again was used as a positive control. In this assay progesterone did not significantly increase penetrating ability at 1 cm in contrast to the donors but it did significantly increase the proportion of cells penetrating at 2 cm the same as donors (figure 6-6).

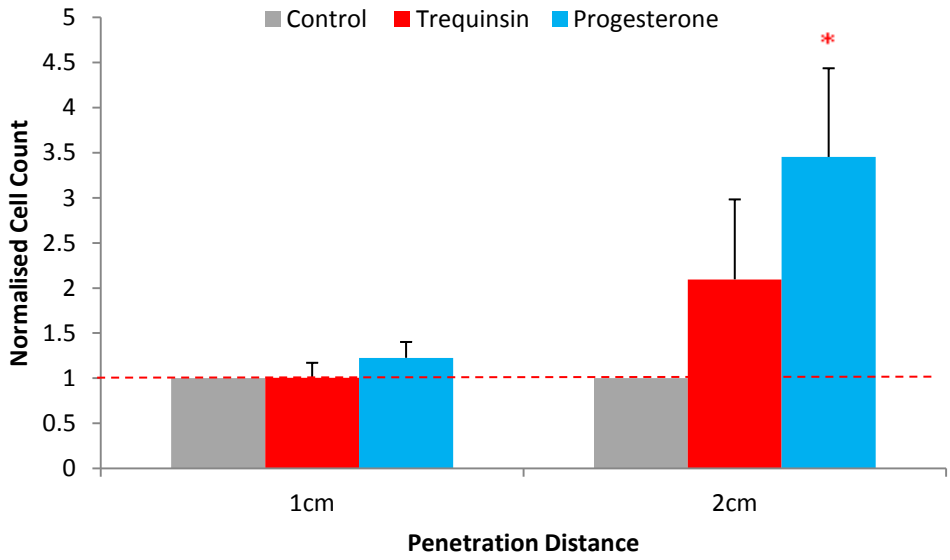


Figure 6-6. Agonist induced sperm penetration into viscous medium. Sperm penetration into viscous medium upon application of 40µM Trequinsin Hydrochloride or 3.6µM progesterone as measured by counting cells at 1 cm and 2 cm. The number of sperm penetrating to 1 cm and 2 cm was assessed and normalised to values in untreated controls (red dashed line). All values are the mean ± SE (n=9 experiments performed with 9 different patient samples). *- indicates a significant difference ($P \leq 0.05$), ***- indicates a significant difference ($P \leq 0.005$) from the control, as assessed by non-parametric analysis Mann Whitney U test.

6.3 Discussion

Trequinsin hydrochloride enhances motility, assessed by CASA, and increases penetration into a cervical mucus substitute

A variety of cellular functions are regulated by cyclic guanine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP) levels (Richter et al., 1999). Cyclic nucleotide functions are dependent on a dynamic intracellular equilibrium between synthesis and degradation (Rossi et al., 1985). The use of nonselective PDE inhibitors have been shown to affect capacitation (Visconti et al., 1995c, deLamirande et al., 1997), sperm motility (Tesariki et al., 1992, Jaiswal and Majumder, 1996) and acrosome reaction (Tesarik et al., 1992).

Trequinsin hydrochloride is a selective potent inhibitor of cGMP-inhibited phosphodiesterase (PDE3). In this study it significantly increased progressive motility and hyperactivation in non-capacitated samples. Lefievre *et al* (2002) identified PDE3A isoforms in ejaculated human spermatozoa however, immunocytochemistry experiments indicated it to be localised to the postacrosomal segment of the sperm head (Lefievre et al., 2002). This same group tested the involvement of PDE3 in sperm function using Milrinone (a selective PDE3 inhibitor) which increased cAMP levels in sperm but not to the extent to induce capacitation or have any effect on motility or tyrosine phosphorylation (Lefievre et al., 2002). However, the data presented in this study contradicts these results with dramatic increases in motility and hyperactivation as a result of treatment with Trequinsin. The positioning of PDE3 at the postacrosomal region of the sperm head does not suggest a role in sperm motility and from the work of Lefievre *et al* (2002) it is reported that the use of PDE3i alone does not increase cAMP levels enough to induce capacitation or alterations in sperm motility (Lefievre et al., 2002). The results from the Flexstation identified Trequinsin as being a compound that increased $[Ca^{2+}]_i$ levels of sperm to an equivalent concentration of that seen after treatment with progesterone suggesting that capacitation may have been triggered in

these sperm cells (Baldi et al., 1991). Elevation of $[Ca^{2+}]_i$ is essential for changes in flagella function as a manifestation of capacitation (Lishko et al., 2012b). This knowledge led to the possibility that Trequinsin was functioning not only through PDE3 but through another signalling pathway. This theory was tested through the use of the patch clamp technique (performed by Steven Mansell) and identified Trequinsin as having activation kinetics almost identical to progesterone (data not shown) suggesting that Trequinsin may act via the CatSper cation channel. Activation of CatSper, by Trequinsin, would lead to an elevation in $[Ca^{2+}]_i$ and promote hyperactivated motility (Mannowetz et al., 2013), both of which are reported in this study. However, cells that had previously been capacitated before exposure to Trequinsin exhibited a time dependent decrease in motility especially in the percentage of hyperactivated cells. Human sperm flagella are long and thin (40μM and <2μM respectively) and because diffusion is inversely proportional to the area through which a substance diffuses it takes molecules many seconds to traverse through the flagellum from the mid-piece to the end-piece (Lishko et al., 2012b). Thus, ATP produced in the mitochondria of the midpiece, which is needed for sperm motility, especially hyperactivation, takes a long time to reach the end of the flagellum (Lishko et al., 2012b). To be able to compensate for this delay, flagella movement especially at the tip of the sperm tail is generated through glycolysis (Miki et al., 2004, Mukai and Okuno, 2004, Williams and Ford, 2001). Glucose is required to sustain an optimal ATP concentration in human sperm motility and is essential in supporting hyperactivated motility (Williams and Ford, 2001). Human sperm obtain a high proportion of their energy from glycolysis of glucose to lactate and a marked decline in vigorous motility was identified when sperm cells were incubated over long periods of time (Williams and Ford, 2001, Chulavatnatol and Haesungcharern, 1977). This decrease in motility was attributed to the stress of prolonged incubation which resulted in a decreased concentration of ATP and ADP.

The use of Trequinsin under capacitating conditions would have required an instantaneous release of ATP to increase and sustain hyperactivation of cells that had previously been capacitated. Williams and Ford (2001) accounted for leakage of adenine nucleotides from dead/dying cells concluding that the loss of ATP/ADP wasn't owing to cell death (Williams and Ford, 2001). Results from this study also showed that Trequinsin did not alter sperm vitality suggesting that the decrease in motility observed under capacitating conditions was not because the compound is detrimental to sperm survival. This same effect had previously been observed with use of caffeine to artificially stimulate motility resulting in an increased demand for energy (Chulavatnatol and Haesungcharern, 1977). If the glycolysis of endogenous substrate is not fast enough the result is a depletion in ATP level detrimental to motility (Chulavatnatol and Haesungcharern, 1977), an effect that was noted in both donor and patient samples which had been capacitated before treatment. However, supplementation of glucose after incubation in all studies mentioned re-stored motility to normal levels suggesting this detriment is not permanent (Miki et al., 2004, Mukai and Okuno, 2004, Williams and Ford, 2001, Chulavatnatol and Haesungcharern, 1977).

Similarly to progesterone (Alasmari et al., 2013b), Trequinsin initiated an increase in penetrating ability of sperm through a cervical mucus substitute, when under non-capacitating conditions. This result supports the determination of Trequinsin hydrochloride functioning via the CatSper channel. This compound has the potential of being beneficial therapeutically in increasing sperm motility however, further research needs to be conducted to confirm the motility effects observed under capacitating conditions are not irreversible and to see whether further supplementation with glucose prevents this effect.

CHAPTER 7

Investigating the effects of novel ion channel compounds on sperm motility

7.1 Introduction

Discovery of new drugs targeting sperm motility to facilitate conception is particularly challenging. Two commonly used methods in drug discovery are computational chemistry and molecular modelling, however HTS has broadened these approaches to allow for identification of hits through screening extensive chemical or focused libraries (Miller, 2006).

Sperm are highly complex systems that rely on post- translational modifications as their primary signalling mechanism. Ionic gradients and ion channels are fundamental resources allowing changes in electrical potential and the concentration of second messengers (Visconti et al., 2011). They regulate intracellular signalling pathways and control physiological changes within the cell (Darszon et al., 2011). Ca^{2+} plays a pivotal role in fertilisation and plays crucial roles in maturation, motility and the acrosome reaction in invertebrate and vertebrate spermatozoa (Yanagimachi, 1994). Over the past 10 years the fundamental role of ion channels and particularly Ca^{2+} to sperm physiology has been consolidated (Kirichok et al., 2006, Darszon et al., 2008). Intracellular Ca^{2+} in sperm appears to be regulated by at least two processes; mobilisation of stored Ca^{2+} in the neck/midpiece region and/or through Ca^{2+} permeable ion channels and transporters (Ho and Suarez, 2001, Ren et al., 2001, Costello et al., 2009).

Ion channels are integral proteins on the membrane of the cell that regulate ion transmission across the cell (Mok and Brenk, 2011). Ion channel targets remain unexploited in drug development with only 10% of drugs currently on the market known to bind to ion channels (Terstappen et al., 2010). Two sperm specific ion channels, CatSper and KSper (Slo3/Slo1) have been identified as the primary spermatozoal ion channels (Ren et al., 2001, Mannowetz et al., 2013, Santi et al., 2010, Schreiber et al., 1998). CatSper is a pH-regulated Ca^{2+} channel that is present on the

flagella of human sperm. Recently, Ca^{2+} induced entry by progesterone has been identified as being via CatSper channels (Lishko et al., 2011, Strunker et al., 2011). CatSper are crucial to sperm motility and are fundamental to male fertility (Ren et al., 2001).

The knowledge of the importance of ion channels and in particular Ca^{2+} in sperm function led to the development of an ion channel assay based on a HTS approach. The HTS assay used was similar to that described for screening the chemogenomics library (chapter 5) however, this assay utilised an in-house ion channel focussed library assembled by the UoD DDU. This library of putative ion channel ligands was assembled from the ChEMBL database and from this library 3312 compounds were screened (Mok and Brenk, 2011). The HTS assay using a Flexstation microplate reader measured the agonist responses of the screened compounds compared to progesterone as a putative control. 70 of the compounds from the primary screen were examined further for >90% purity as demonstrated by chromatography- mass spectrometry (LCMS), reducing the number of compounds to 48 that evoked a concentration dependent increase in $[Ca^{2+}]_i$. The molecular and chemical structures of these compounds were further examined and 14 ion channel compounds were selected to undergo in-depth spermatozoa motility assessments.

The 14 compounds will be identified as A1- H1 and A2- F2. Of the 14 compounds subjected to motility analysis the 5 most promising compounds (A1-E1) will be discussed in detail in this chapter with motility data for the remaining 9 (F1-H1 & A2-F2) being accessible in the appendix.

7.2 Experimental Procedure

Motility analysis was conducted on spermatozoa from the 40% fraction, under non-capacitating conditions, after DGC, from donor spermatozoa. This fraction was used as a surrogate for a patient sample (chapter 3). The 80% fraction, from a donor sample, under capacitating conditions, was evaluated to see if the compounds were capable of increasing motility in the high-density portion of strongly motile sperm (Oaconnell, 2003). Non-capacitated samples were left in the incubator at 37^{o}C for 15 min before treatment with the compounds whereas capacitated samples were left in the 5% CO_2 incubator for 2.5 hrs before treatment with the compounds (for further details see chapter 2). Analysis was conducted at 30 minute intervals for a period of 3 hrs. We will only be discussing total motility, progressive motility in detail in this chapter. The results for VCL, ALH and hyperactivation are available in the appendix.

Kremer mucus penetration tests, acrosome reaction analysis and vitality tests were conducted in an identical manner to that described in chapter 6.

7.2 Results

7.2.1 Motility assessment by CASA of five hit compounds

7.2.1.1 *Effect of 5 compounds on motility of 40% fraction in non-capacitating buffer*

A1 exhibited no significant differences to control in any of the motility parameters evaluated by CASA when the spermatozoa from the 40% fraction were evaluated under non-capacitating conditions (appendix). Total motility and progressive motility were unaffected by the compound at any of the time points analysed (figure 7-1 A&B).

Spermatozoa incubated with B1 had a similar response to A1 with total motility and progressive motility exhibiting very little change in flagella motility between the treated sample and control (figure 7-1 A&B). The effects of C1, D1 and E1 on motility were very similar to that of A1 and B1with no significant changes seen in treated cells (figure 7-1 A&B).

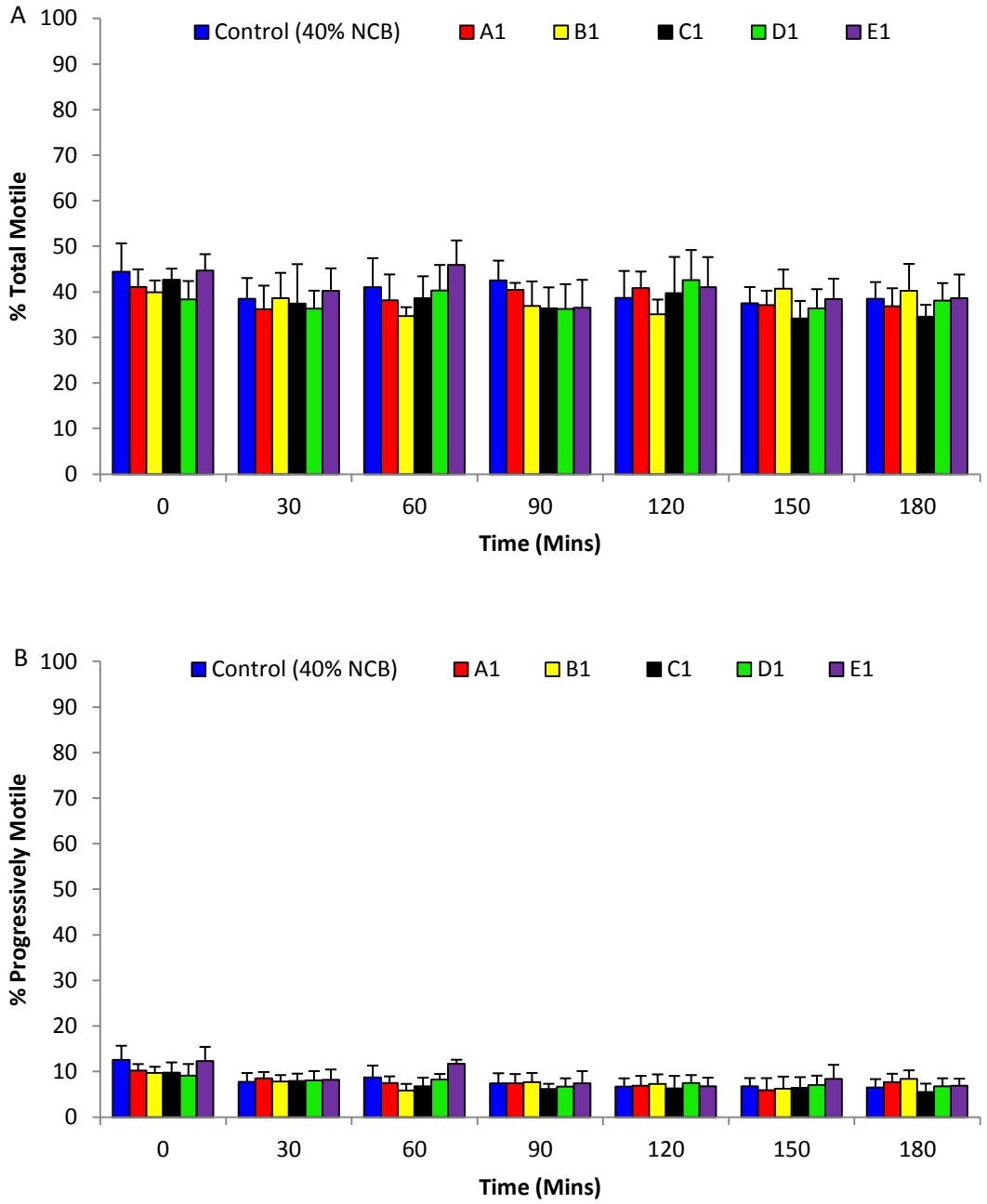


Figure 7-1. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % total motile and **(B)** % progressive motility**.** The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

7.2.1.2 *Motility resulting from exposure to 5 novel compounds in the 80% fraction after capacitation*

Exposure of spermatozoa from the 80% fraction, which had been capacitated before treatment, exhibited no significant changes from control for either total motile or progressive motility after treatment with A1, B1, C1 or D1 (appendix, figure 7-2). However, sperm incubated for 60 min with E1 were found to be significantly more progressively motile than control cells ($P \leq 0.05$) (figure 7-2) although, this was the only time point to exhibit significant increases. Examination of motility by use of CASA did not highlight any compounds that had a sustained, positive, significant effect on motility however; none of the compounds appear to be having a negative effect as was seen from compounds identified from the Chemogenomics screen (chapter 5).

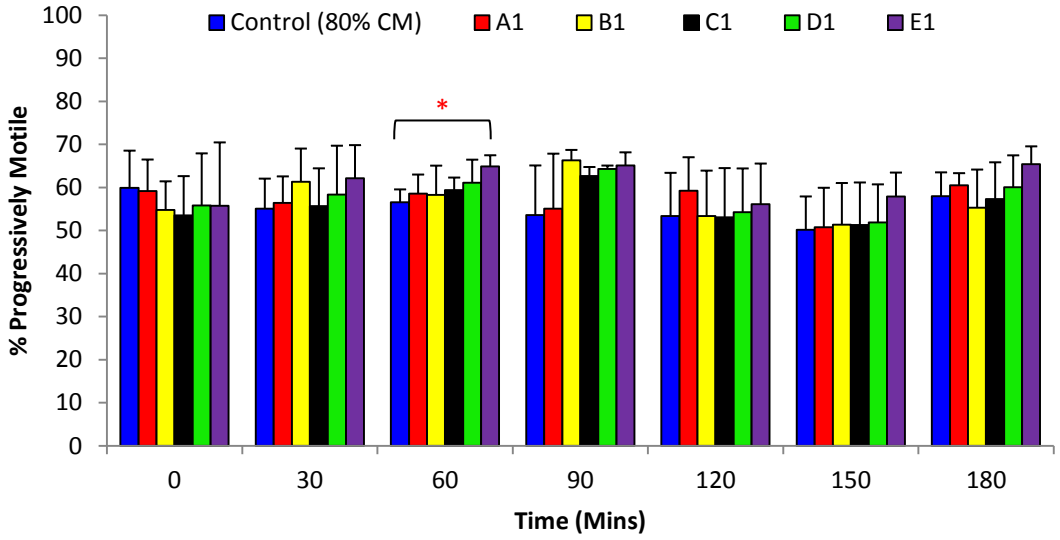


Figure 7-2. Expression of basal (control) motility values compared to treated samples in capacitating media (CM), 80% fraction for % progressive motility. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase ($P \leq 0.05$) between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

7.2.2 Evaluation of sperm motility into artificial viscous medium (Kremer test)

To further evaluate sperm motility a functional assay that measures penetrating ability of cells into a viscous substrate (methylcellulose – alternative to cervical mucus) was conducted (Kremer, 1965, Ivic, 2002). To determine whether the motility response following the addition of agonists leads to impaired or improved penetration into viscous medium, donor samples were incubated with agonists for 1hr before cell penetration was analysed.

The initial analysis was conducted on the 80% fraction following capacitation. Once again progesterone was used as a positive control alongside the treated compounds (see chapter 6.3.2). Exposure of cells prepared in CM to C1, D1 and E1 failed to increase cell penetration into methylcellulose. Stimulation with B1 and 3.6μM progesterone significantly enhanced penetration into viscous medium at distances of both 1 cm and 2 cm (B1 $P \leq 0.005$, progesterone $P \leq 0.001$; figure 7-3 A&B). A1 significantly decreased penetration at 1 cm ($P \leq 0.05$) however; it appeared to increase penetration at 2 cm although this did not reach statistical significance (figure 7-3 A&B).

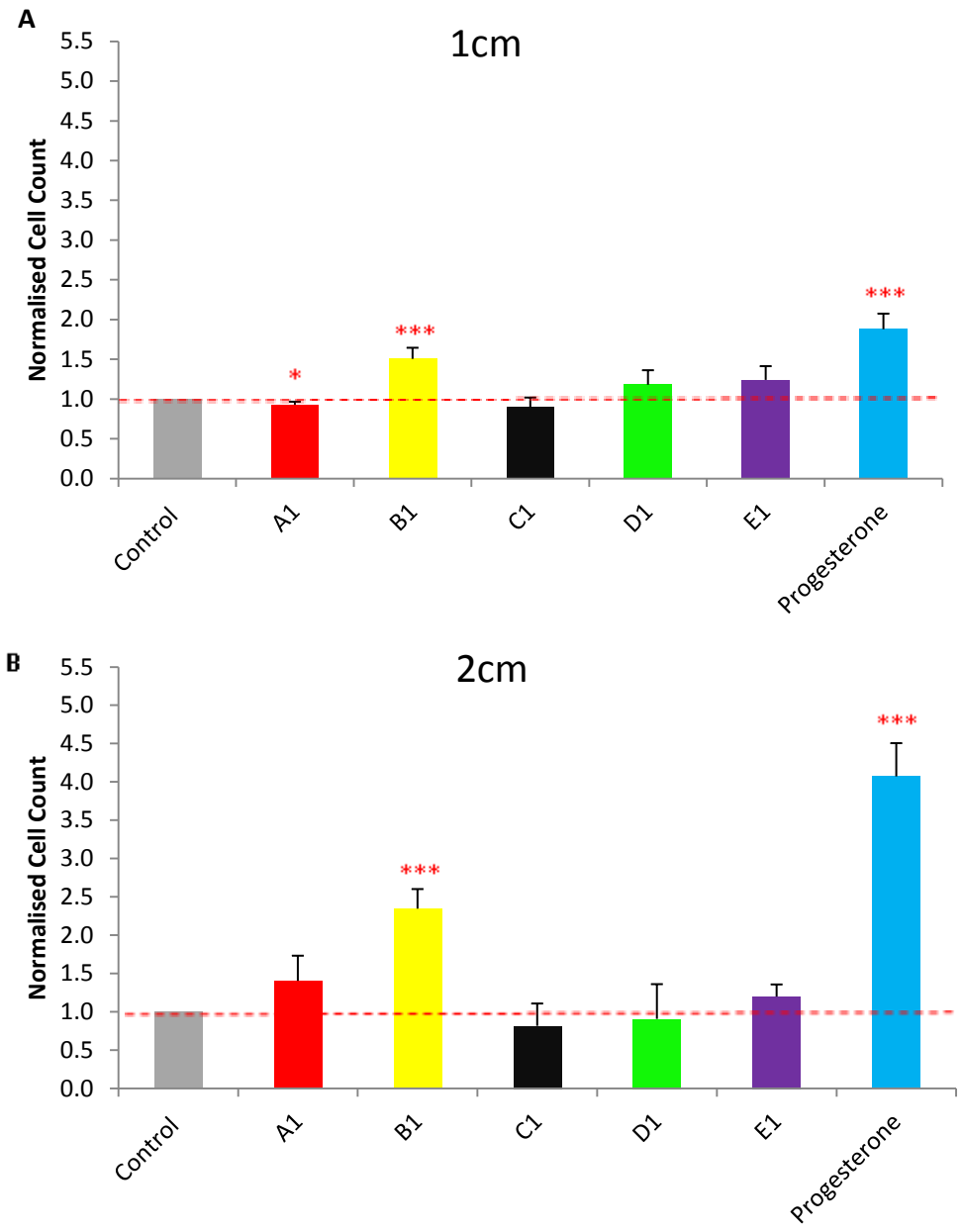


Figure 7-3. Agonist induced penetration of cells into viscous medium. Cells from the 80% fraction under capacitating conditions were evaluated for the number of sperm penetrating to 1 cm **(A)** and 2 cm **(B).** The cells were counted and normalised to values in untreated controls (red dashed line). Agonists include: A1, B1 (n=5 experiments with 5 different donors), C1, D1 (n=3 experiments with 3 different donors), E1 (n=5 experiments with 5 different donors) and progesterone (n=11 experiments with 6 different donors). All values are the mean ± SE. *- indicates a significant difference from the control, considered to be $P \leq 0.05$, ***- indicates significance of $P \leq 0.005$ & $P \leq 0.001$ as assessed by non-parametric Mann Whitney U test.

Sperm motility, when measured by CASA, was not found to increase as a result of agonist exposure (figure 7-1& 7-2) however, the penetration assay found B1 and progesterone significantly enhanced penetrating ability of sperm cells when under capacitating conditions (figure 7-3 A&B). To examine whether this effect was the same in our surrogate patient sample (40% fraction under non-capacitating conditions) the penetration assay was conducted on A1, B1, E1 and progesterone. It was decided to examine A1 under these conditions due to the increase in penetration seen at the 2 cm position under capacitating conditions (figure 7-3B) and E1 was selected following a significant increase in progressive motility being exhibited through CASA analysis (figure 7-2). Sperm cells exposed to C1 and D1 were not found to enhance motility or penetrating ability through CASA analysis or the Kremer assay and for this reason were not evaluated under these conditions. Sperm cells exposed to A1 and E1 showed no significant changes in penetrating ability compared to control at either distance. B1 and progesterone were both found to enhance penetrating ability at 1 cm however; this was not a significant result (figure 7-4A). At the 2 cm position B1 increased sperm cell penetration however; the results between donors were highly variable and did not produce a significant result. Progesterone produced a significant increase in the number of sperm cells able to penetrate the viscous medium at the 2 cm position under these conditions ($P \leq 0.05$; figure 7-4B).

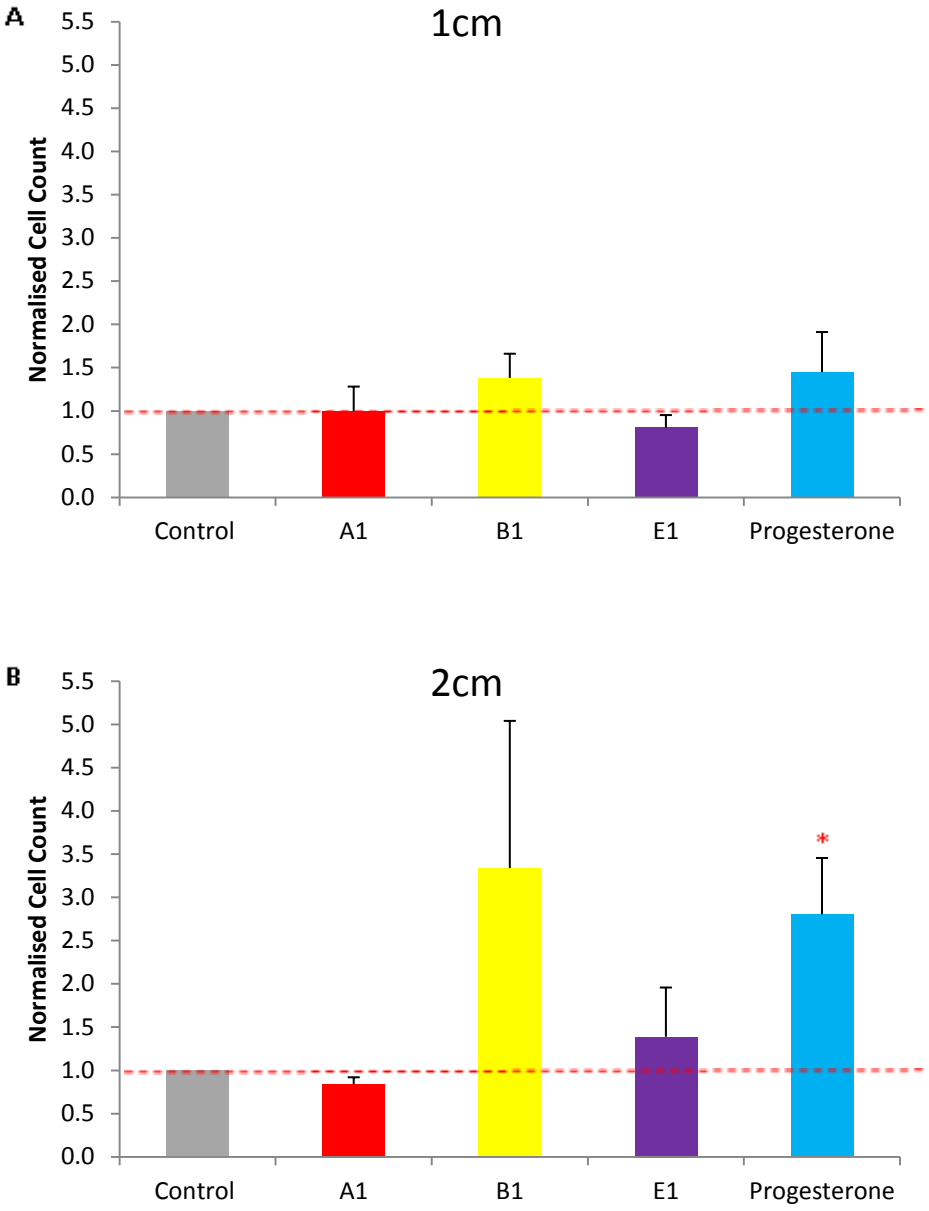


Figure 7-4. Agonist induced penetration of cells into viscous medium. Cells from 40% fraction under non-capacitating conditions were evaluated for the number of sperm penetrating to 1cm **(A)** and 2cm **(B).** The cells were counted and normalised to values in untreated controls (red dashed line). Agonists include: A1 (n=4 experiments with 4 different donors), B1 (n=5 experiments with 5 different donors), E1 (n=4 experiments with 4 different donors) and progesterone (n=18 experiments with 5 different donors). All values are the mean ± SE. ***-** indicates a significant difference from the control, considered to be $P \leq 0.05$ as assessed by non-parametric Mann Whitney U test.

7.2.3 Evaluating acrosome reaction in response to agonist stimulation

The Flexstation assay highlighted C1 and D1 as increasing intracellular Ca^{2+} however, this increase in Ca^{2+} is does not appear to affect motility measured, by CASA or the Kremer mucus penetration test. This raises the question as to what this increase in $[Ca^{2+}]_i$ can be attributed to. The knowledge of a Ca^{2+} store in the acrosomal region (Walensky and Snyder, 1995) led to the examination of the acrosome of cells after exposure to the aforementioned compounds.

The mean percentage of sperm with reacted acrosomes was significantly increased compared to control when cells were exposed to 1% DMSO ($P \leq 0.05$; figure 7-5 A&B). The response to 1% DMSO was analysed due to these DDU compounds being in combination with DMSO. C1 induced the acrosome reaction in 42% of sperm cells which was a significantly higher percentage of cells compared to control, however, this result was not significantly different to the DMSO induced reaction indicating that this significant increase was most likely not a response solely of the agonist in question (figure 7-5A). D1 provoked the acrosome reaction in 59% of sperm cells which was a significantly higher proportion of cells than both the control and DMSO treated ($P \leq 0.05$; figure 7-5B). This result suggests that D1 could potentially be inducing a premature acrosome reaction which would be detrimental to the fertilising potential of spermatozoa exposed to this compound. To ensure that the experiment was running successfully the effect of Ca^{2+} ionophore (A23187), a known inducer of the acrosome reaction (Aitken et al., 1993), was conducted in parallel to agonists. In both sets of experiments Ca^{2+} ionophore significantly enhanced the percentage of acrosome reacted cells when compared to control ($P \leq 0.05$; figure 7-5A, $P \leq 0.001$; figure 7-5B), DMSO ($P \leq 0.05$; figure 7-5A, $P \leq 0.001$; figure 7-5B), C1 ($P \leq 0.05$) and D1 ($P \leq 0.05$) independently (figure 7-5 A&B).

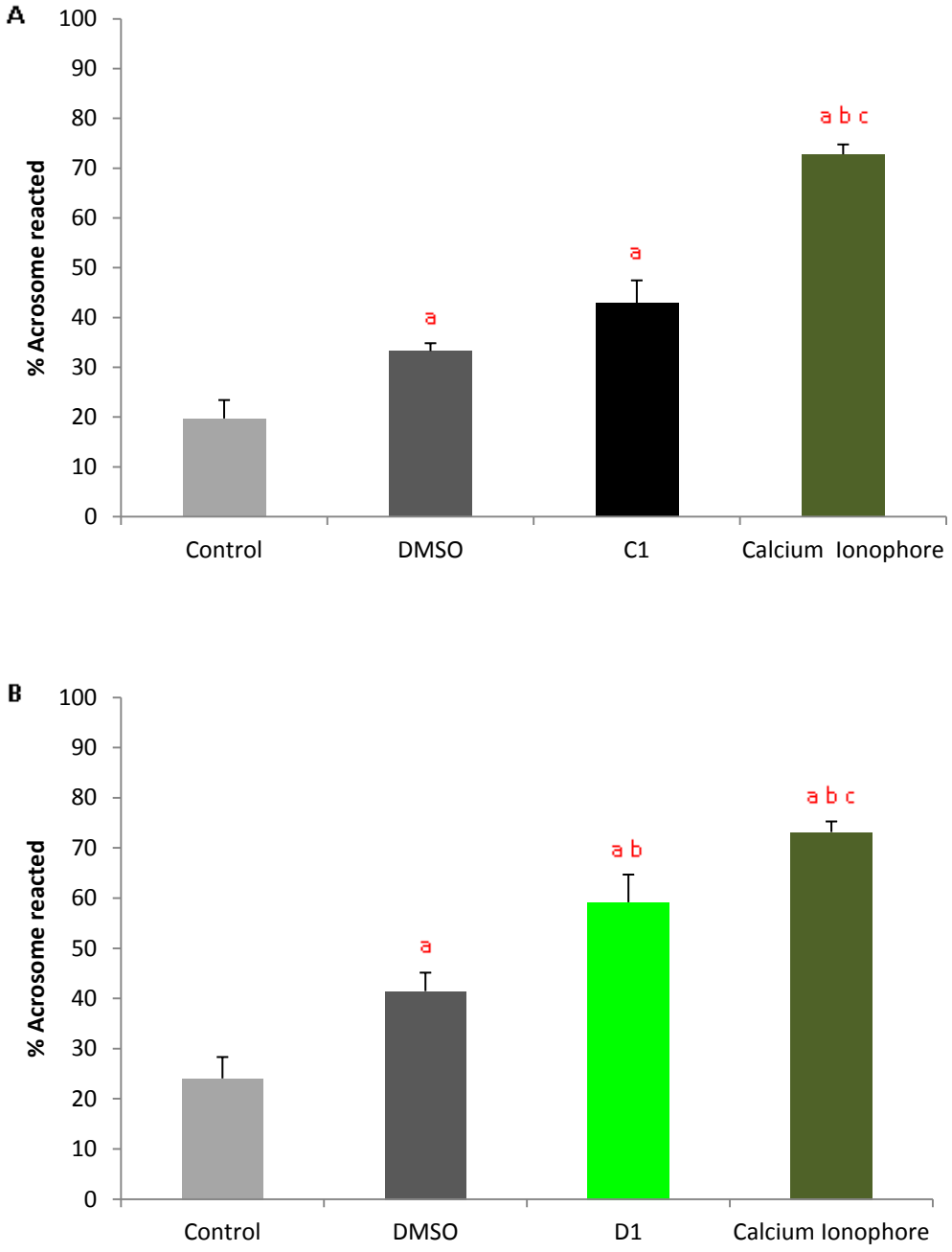


Figure 7-5. Agonist induced acrosome reaction. Staining with PSA-FITC allowed for evaluation of the percentage of sperm cells that become acrosome reacted in response to C1 **(A)** and D1 **(B).** All values are the mean ± SE, n=5 for all experiments. Significance was considered as $P \leq 0.05$ indicated by a (significantly different from control), b (significantly different form DMSO) and c (significantly different to agonist C1 or D1). Statistical significance evaluated by T-test or non-parametric Mann Whitney U test.

7.2.4 Vitality assessment of sperm cells exposed to agonists

To further identify where this increase in $[Ca^{2+}]_i$ measured by the Flexstation assay may have come from or could be attributed to the Hypo-osmotic swelling test (HOS) was conducted to test vitality after exposure to the agonists. It is known that as cells die they tend to show an increase in $[Ca^{2+}]_i$ as a result. To be able to assess whether this increase seen in the Flexstation was a result of the cells dying the HOS test was conducted on cells stimulated by both C1 and D1. Capacitated sperm cells were exposed to either C1 or D1 for a period of 3 hrs, equivalent to the amount of exposure assessed in the CASA motility assay, and the number of living cells assessed.

The HOS test identified that neither C1 nor D1 appeared to have a detrimental effect on sperm vitality. The result for C1 indicated a mean of 93.5% of living cells with the results ranging from 85% - 99% from the 3 donor samples assessed (figure 7-6). Sperm cells exposed to D1 showed similar results to C1 with a mean of 95% living cells and a range of 90% - 98% from the 3 samples assessed (figure 7-6). Neither C1 nor D1 were significantly different from control (figure 7-6) suggesting that neither of the compounds were toxic to sperm cells.

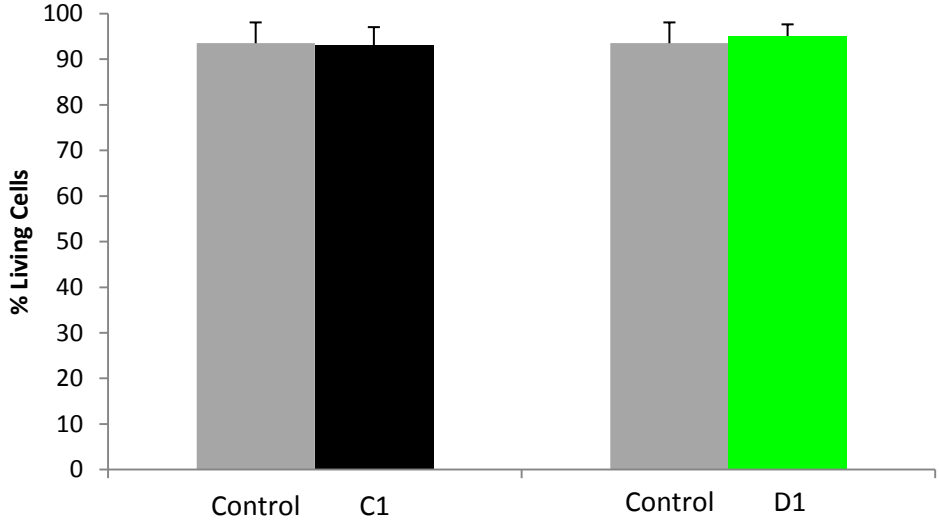


Figure 7-6. Vitality analysis after agonist exposure. C1 (black bar) and D1 (green bar) were assessed for their effects on sperm vitality after incubation with sperm cells for a period of 3 hrs. All values are the mean ± SE, n=3 for each experiment. Neither C1 nor D1 were statistically different from control (grey bars). Analysis was conducted using Pearson's Chi squared test and significance was assessed as $P \leq 0.05$.

7.2.5 Effect of ion channel agonists on patient samples

Analysis of ion channel compounds on motility of donors conducted through CASA analysis did not highlight any compounds that consistently increased any of the motility parameters evaluated (section 7.2.1). However, the Kremer penetration assay identified B1 along with progesterone as significantly stimulating penetration through the cervical mucus substitute methylcellulose (section 7.2.2). To determine whether this was a true agonist response CASA motility analysis and the Kremer penetration assay were conducted on patient samples after exposure to B1.

7.2.5.1 *CASA motility analysis of patient samples*

Patient samples provided by the ACU were incubated with B1 and motility kinematics measured by CASA over a period of 2 hrs. All samples examined were from patients undergoing IVF at the ACU and the results from each sample will be assessed independently (table 7.1).

Incubation with B1, when compared with control, resulted in a huge variation in responses, in all parameters, between the 9 patients analysed. Significant increases were found at points in every parameter evaluated but none of these significant increases were consistent between samples (table 7.1). The only parameter that contained a sustained statistical increase over the 2 hr time course was found for the percentage of total motile cells, however, this was limited to only one sample (P8, table 7.1). P1 and P6 showed significant decreases in VCL compared to control that were sustained for the 2 hrs evaluated. The predominant result after stimulation with B1 in all parameters was no significant change from control which corroborates the data from the donor analysis with this compound.

B1												
	VCL				LIN				ALH			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	×	×	×	×	–	✓	–	–	–	×	×	–
P2	–	✓	×	–	–	–	×	✓	–	–	–	×
P3	–	–	–	–	–	✓	–	✓	–	×	×	×
P4	✓	–	–	–	–	✓	–	–	✓	–	–	–
P5	–	–	–	–	–	–	–	–	–	–	–	–
P6	–	×	×	×	–	–	–	–	–	–	–	–
P7	–	–	–	–	–	–	–	–	–	–	–	–
P8	–	–	–	✓	–	–	–	✓	–	✓	–	–
P9	–	–	–	–	–	–	–	–	–	–	–	–
	Total Motile				Progressive Motility				Hyperactivation			
Patient No.	0	30	60	120	0	30	60	120	0	30	60	120
P1	–	×	–	–	–	–	×	–	×	–	–	×
P2	–	–	–	✓	–	–	×	✓	–	–	✓	–
P3	–	×	✓	–	–	✓	×	–	–	–	–	–
P4	–	–	–	–	–	–	–	–	–	–	–	–
P5	–	–	–	–	–	–	–	–	–	–	–	–
P6	–	–	–	×	–	–	✓	×	–	–	–	–
P7	–	–	–	–	–	–	–	–	–	–	–	–
P8	–	✓	✓	✓	–	–	–	✓	–	–	–	✓
P9	–	–	–	×	–	–	–	–	–	–	–	–

Key

✓=Increase

× = Decrease

– = No change

Table 7.1 Identification of motility kinematics, measured by CASA, from patient samples after exposure to B1. Motility parameters after stimulation with the agonist B1were evaluated from 9 patients undergoing IVF at Ninewells ACU. The table identifies the 6 motility parameters evaluated for each patient over a period of 2 hrs. Statistical significance (see key) was determined from comparison with control ± SD. If the agonist response was greater than or less than control it was considered as a significant result.

7.2.5.2 *Assessing functional motility of patient samples stimulated by B1*

Section 7.2 identified that treatment with B1 and progesterone significantly enhances penetration of donor cells into methylcellulose (B1 $P \leq 0.005$, Progesterone $P \leq 0.001$, n=5 figure 7-3 A&B). To ensure this result was not only valid in WHO normal donor samples the same assay was conducted on patient samples. The patients were all undergoing IVF at the ACU at Ninewells hospital. The results from this assay were consistent with the donor assay with cells exhibiting significantly increased penetrating ability after incubation with B1 and progesterone ($P \leq 0.05$ at 1 cm, $P \leq 0.005$ at 2 cm; figure 7-7). However, B1 was only found to show a significant increase in the number of cells penetrating to the 2 cm position ($P \leq 0.05$; figure 7-7) instead of at both positions as seen with donor samples (figure 7-3).

When the behaviour of sperm swimming within viscous medium was investigated using CASA, neither B1 nor progesterone produced significant alterations in the speed of progression through the viscous medium (appendix). This observation is consistent with the effects of B1 seen in standard medium (figure 7-1 & 7-2).

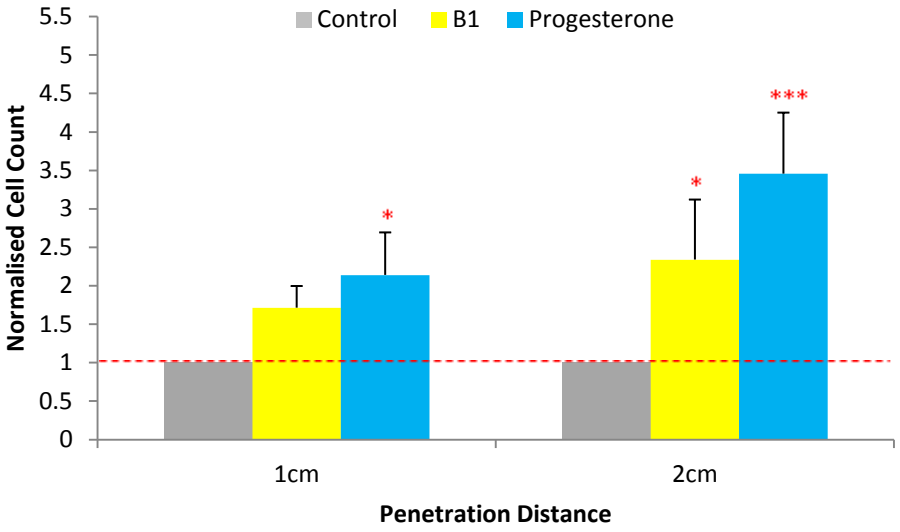


Figure 7-7. Agonist induced sperm penetration into viscous medium. Sperm penetration into viscous medium upon application of 10µM B1 or 3.6µM progesterone measured by counting cells at 1 cm and 2 cm. The number of sperm penetrating to 1 cm and 2 cm was assessed and normalised to values in untreated controls (red dashed line). All values are the mean ± SE (n=9 experiments performed with 9 different patient samples). *- indicates a significant difference ($P \leq 0.05$), ***- indicates a significant difference ($P \leq 0.005$) from the control, as assessed by non-parametric analysis Mann Whitney U test.

7.3 Discussion

The aims of this study were: 1) to examine whether the ion channel compounds increased sperm motility when evaluated by CASA analysis, 2) analyse whether agonist treated sperm were capable of penetrating viscous medium more effectively than untreated controls, 3) to establish the effects of agonists on sperm vitality and the ability to undergo the acrosome reaction and 4) after identifying promising compounds, to examine the robustness of the results by applying agonists to patient samples.

B1 activation promotes sperm penetration into viscous medium but does not influence motility kinematics specifically

The results from this chapter did not identify any ion channel compounds that consistently increased motility parameters, of donor or patient sperm, over a 3hr time-course when analysed by CASA. This prompted examination of these agonists using an alternative motility assay, the Kremer mucus penetration test. Interestingly, the results from this assay identified that B1 and progesterone significantly enhanced penetrating abilities of sperm through a cervical mucus surrogate (methylcellulose) although, it did not significantly change any motility kinematic parameters.

A previous study has described that direct activation of CatSper channels by progesterone has insignificant effects on hyperactivation when assessed by CASA analysis, however, progesterone was found to strongly enhance sperm penetrating abilities into methylcellulose (Alasmari et al., 2013b). This effect is similar to this study with the ion channel compound B1 showing insignificant effects on motility and hyperactivation when evaluating cells under both non-capacitating and capacitating conditions. However, this same compound significantly enhances spermatozoa's penetrating ability into viscous medium under both conditions. The Flexstation assay identified B1 as increasing $[Ca^{2+}]_i$ in sperm cells similarly to progesterone. These

findings could indicate that B1 may be acting via the sperm specific, Ca^{2+} permeable, CatSper cation channel in a similar manner to progesterone, although, further motility analysis needs to be conducted making use of CatSper ion channel blockers to confirm this theory. Stimulation of human sperm with progesterone has been shown to activate CatSper situated in the flagellum (Strunker et al., 2011, Lishko et al., 2011). The associated increase in $[Ca^{2+}]_i$ is amplified and propagated forward by CICR at the sperm neck (Costello et al., 2009, Olson et al., 2010, Lefievre et al., 2012). Previous studies on animals and human have reported active Ca^{2+} propagation from the tail to the midpiece and head in response to varying stimuli (Xia et al., 2007, Suarez et al., 1993). This Ca^{2+} propagation has an active role in the regulation of sperm behaviour. The theory behind B1 possibly acting via CatSper instead of acting via activation of Ca^{2+} stores results from previous work identifying that agonists that actively induce Ca^{2+} increase only from Ca^{2+} stores leads to a visible increase in hyperactivation as measured by CASA, whereas, the use of CatSper blockers on the same compounds resulted in no visible alteration in hyperactivation when measured by CASA (Alasmari, 2013). This information leads us to surmise that if B1 solely activated release of Ca^{2+} from internal stores you would have seen an increase in hyperactivation using CASA, which is not the case in this study. This suggests to me that it is more likely that B1 may act on CatSper with motility findings reported in this chapter being similar to that of this previous study with regard to compounds such as progesterone. Progesterone was found to regulate CatSper without showing an increase in hyperactivation that was measurable by CASA, similarly to B1. However, treated cells did exhibit significant increases when it came to the ability of spermatozoa to penetrate through methylcellulose (Alasmari, 2013, Alasmari et al., 2013b) which is a similar trait to that observed when spermatozoa are treated with B1. To be able to confirm this theory the next step for B1 would be to attempt to make use of known CatSper blockers on treated cells and measure $[Ca^{2+}]_i$

alterations and motility through both CASA and the Kremer mucus penetration test see if you still see the increase in penetrating ability.

It has been reported that for sperm to be effective at penetrating through mucus they require the amplitude of lateral head movements to be ≥2.5μM but <7.0μM (Bjorndahl et al., 2010) . Similarly Tesarik et al described that for human sperm to be capable of penetrating the cumulus matrix they required small amplitudes of lateral head displacement with a predominantly linear movement but an increased beat cross frequency (Tesařík et al., 1990). Alasmari et al describe the main functional effect of activating CatSper is an enhanced penetration into viscous medium (Alasmari et al., 2013b) which is an effect exhibited by B1 treated sperm. This enhanced penetration into viscous medium without enhanced motility kinematics, when measured by CASA, may result from the limitations of the CASA system which only measures movement of the sperm head and would be unable to distinguish subtle alterations in flagella beat that would enhance entry into viscous medium (Alasmari et al., 2013b). A group had previously investigated the effects of a caged progesterone analog (a known CatSper activator) on flagella bending of human sperm and they identified that after the progesterone activated CatSper it induced changes in curvature at the distal flagellum (Servin-Vences et al., 2012), these changes would not have been recognised by CASA owing to its inability to measure anything but alterations in movement at the sperm head. This would explain why no visible changes were noticed in motility when measured by CASA alone.

This compound has the potential to be beneficial in a clinical setting acting to increase penetrating ability of spermatozoa with no detrimental effects on motility. This could be beneficial in treatments such as IUI in aiding the sperm to swim through and penetrate the zona pellucida and oocyte vestments to bind to the oocyte.

CHAPTER 8

Investigating the incidence of sub-fertile patients, from andrology clinics, who could benefit clinically from sperm stimulants

8.1 Introduction

Standard semen analysis is a subjective technique and has been found to be associated with large inter-laboratory variation making it difficult to predict accurate values (Larsen et al., 2000, Hargreave and Elton, 1983). Previously there had been controversy over the use of WHO reference values for diagnosis of infertility owing to the population they were created from being a 'normal population' of healthy men, rather than from men with proven fertility, raising concerns about the possibility of men conceiving who have semen analysis values below the WHO cut-off values (Lewis, 2007, Jedrzejczak et al., 2008, Ombelet et al., 1997). The current WHO 2010 reference values have now corrected for this and the reference values generated are from healthy donors with known fertility (time to pregnancy ≤12 months) (Cooper et al., 2010). The lower reference limits (LRL) outlined by WHO 2010, especially where motility is concerned (total motility; LRL 40% and progressive motility; LRL 32%), are similar to those proposed by John MacLeod and Ruth Gold in 1951 (Macleod and Gold, 1951b) suggesting that these new values are more robust than previous editions (Publicover and Barratt, 2011).

Sperm motility is one of the fundamental requirements for sperm to be capable of successful fertilisation *in vivo* and *in vitro* (Donnelly et al., 1998). MacLeod and Gold (Macleod and Gold, 1951b) identified that there are clear differences in sperm motility between sub- fertile and fertile men which has since been corroborated by numerous groups (Barratt et al., 2011). For this reason it is one of the key characteristics that is measured through semen analysis to provide diagnostic and prognostic information for natural conception and ART (Publicover and Barratt, 2011). Along with motility, sperm morphology and concentration are also important factors affecting fertility that are measured during a routine semen analysis (Guzick et al., 2001). Proposed treatment plans for male factor infertility are dependent on the results from two independent

semen analyses (two samples produced on separate occasions with a minimum of 2 days and maximum of 7 days abstinence) with IUI being the initial treatment option used in an attempt to alleviate mild male factor infertility (Tournaye, 2012). To be eligible for IUI treatment the female partner has to have a normal fertility status and the unprocessed semen sample must contain a total motile count (TMC) of at least $1x10^6$-$3x10^6$ which corresponds to mild oligoasthenozoospermia and a minimum of $0.8x10^6$ progressively motile cells must be recovered after sperm preparation (Tournaye and Cohlen, 2012, Tournaye, 2012, van Weert et al., 2004). Conception rates through IUI are very poor which reduces its use as the first-line treatment (van Weert et al., 2004, Campana et al., 1996) however, compared with IVF there is good evidence to suggest that IUI is a cost-effective treatment that should be initiated before more invasive and expensive treatments are considered (Goverde et al., 2000, Karande et al., 1999).

The range and distribution of semen profiles of men attending different infertility clinics is considerable. It is difficult, and maybe impossible, to identify a standard patient profile from semen analysis data owing to patients representing different clinical populations i.e. patients are a mixture of primary and tertiary referrals, NHS or private. Although this is the case, using semen analysis data from the two centres as a guide, it is possible to predict the percentage of patients who may benefit from *in vitro* drug stimulation to increase the concentration of motile cells. Publicover and Barratt (2011) constructed a graph using data adapted from Hargreave and Elton (1983) to predict conception according to the concentration of motile cells (figure 8-1) (Publicover and Barratt, 2011, Hargreave and Elton, 1983). This graph identifies reduced conception rates below 2 million motile cells/ml before a plateau occurs where the numbers of motile cells do not have an effect on conception. The area that is important for targeting for novel drug stimulation, according to this graph, would be patients who have less than 2 million motile cells/ml.

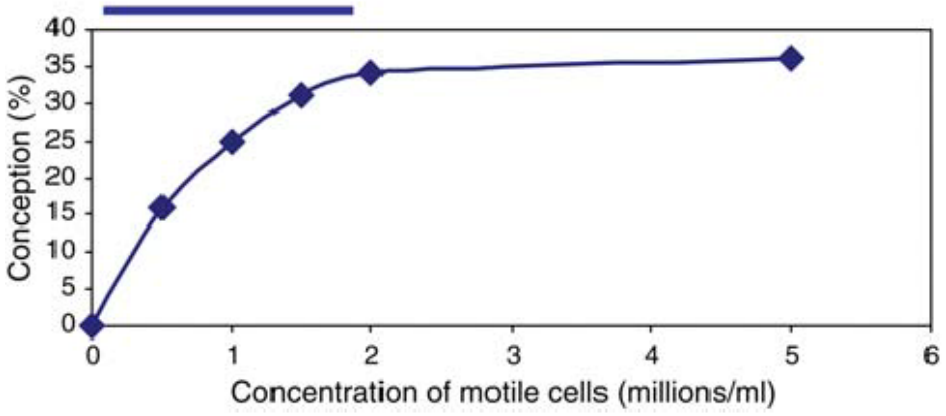


Figure 8-1 Figure courtesy of Publicover and Barratt denoting likelihood of conception with regard to the concentration of motile cells. Data adapted from Hargreave and Elton (1983) depicting a plateau in conception rates with higher concentrations of motile cells. Reduced conception rates are noted below 2 million motile cells/ml (blue bar) this is the target area where improving motility by use of motility enhancing drugs would have most effect on improving fertility (Publicover and Barratt, 2011).

Using the data from Publicover and Barratt (2011) and the knowledge that an unprocessed semen sample must contain a TMC between $1x10^6$- $3x10^6$ to qualify for IUI (Tournaye and Cohlen, 2012), as a first-line treatment, this study aims to identify the benefits of a specific treatment i.e. novel drug therapies that can be used to increase sperm motility. The clinical use of sperm stimulants may be enough to increase the TMC of some patients, who previously were not illegible for IUI owing to a low TMC, to allow them to use less invasive treatments as a first treatment option.

8.2 Experimental design

The analysis evaluated the semen characteristics of 3239 patients from clinic A and 552 patients from clinic B. Patients where all the data was missing were excluded from the study (31 patients' clinic A, 0 patients' clinic B). Patients were divided into categories according to the WHO 2010 reference parameters for semen analysis with patients being classed as oligozoospermic (concentration of <15 million/ml), asthenozoospermic (total motility <40% and/or progressive motility <32%), teratozoospermic (normal forms <4%) or a combination of two or three of these factors, cryptozoospermic (<1 million/ml), normozoospermic (concentration >15 million/ml, total motility >40%, progressive motility >32% and >4% normal forms) and finally azoospermic.

For statistical analysis all parameters were analysed, utilizing frequency distributions and P-P plots (probability plots for assessing how closely two data sets agree), for normality. Semen analysis data from both of the clinics was not found to be normally distributed and after transformation the data remained non-normal. For the purposes of correlation/regression data is not required to be normally distributed, however, assumptions are made about "error" which is the difference between the observed and predicted values (also called residuals). Scatter plots and correlation analysis was conducted on three pairs of variables; 1) % motile with % normal forms, 2) % motile with concentration and 3) % normal forms with concentration. Linear regression analysis was then conducted on these same variables and the "error" was analysed for normality by use of histograms.

Cumulative frequency distributions depicting the concentration of motile cells were generated for the two datasets. Azoospermic patients were excluded from the dataset. To focus the dataset only patients with a concentration of 6 million motile cells per ml (calculated from WHO 2010 lower reference limits: concentration <15 million/ml, total motility <40%) were included in the analysis (clinic A n= 687, clinic B n=175).

8.3 Results

According to the WHO 2010 reference parameters for semen analysis, for clinic A, 56% of the patients were identified to be normozoospermic with 6% exhibiting solely motility dysfunction (asthenozoospermia) and a further 10% showing poor motility combined with either poor sperm morphology, low concentration or a combination of all three factors (teratozoosprmia, oligozoospermia or oligoasthenoteratozoospermia) (figure 8-2). This differed to the data collected from clinic B with only 24% of patients being considered normal and 4% exhibiting isolated asthenozoospermia with a further 25% exhibiting poor motility in combination with other factors (figure 8-3). With the exception of the patients characterised has being normal the highest percentage of patients in both clinics were identified as being teratozoospermic 10% and 22%, clinic A and B respectively (figure 8-2 and 8-3). When the three single parameters (oligozoospermia, teratozoospermia and asthenozoospermia) were compared with one another the correlation between the parameters was found to be highly significant ($P \leq 0.000$) for both clinic A and B although, after visually comparing the parameters in a scatter plot you would not expect this to be a highly significant result (figure 8-4 A-C and 8-5 A-C). Correlation can be statistically significant simply because of the large number of cases, not because there is a meaningful bivariate relationship which appears to be the case for these datasets. Baring this in mind, when looking at the correlations, the linear regression model identifies the percentage of variance explained by the model, which for clinic A is 11%, suggesting that both morphology and sperm concentration influence progressive motility and vice versa. Similarly for the dataset for clinic B linear regression analysis identified that both independent variables (sperm concentration and morphology) contribute to the model of motility 16% of the time. These percentages suggest there is a correlation between the three factors although it is minimal.

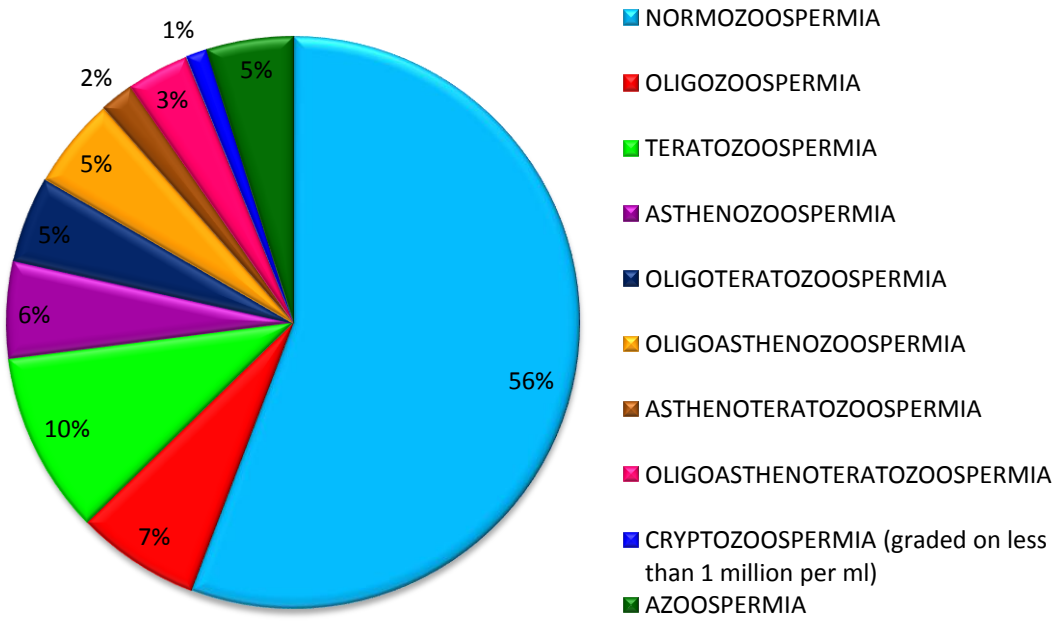


Figure 8-2 Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic A (2002-2003) .

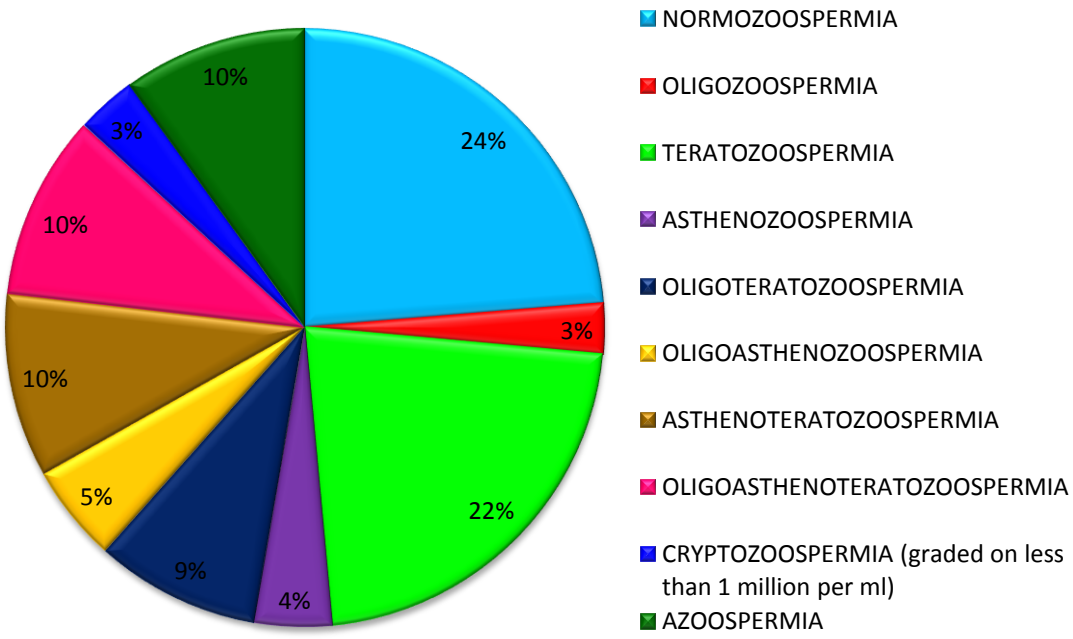


Figure 8-3. Classification of patients according to WHO reference parameters (WHO, 2010e) for semen analysis for patients attending clinic B (2011)

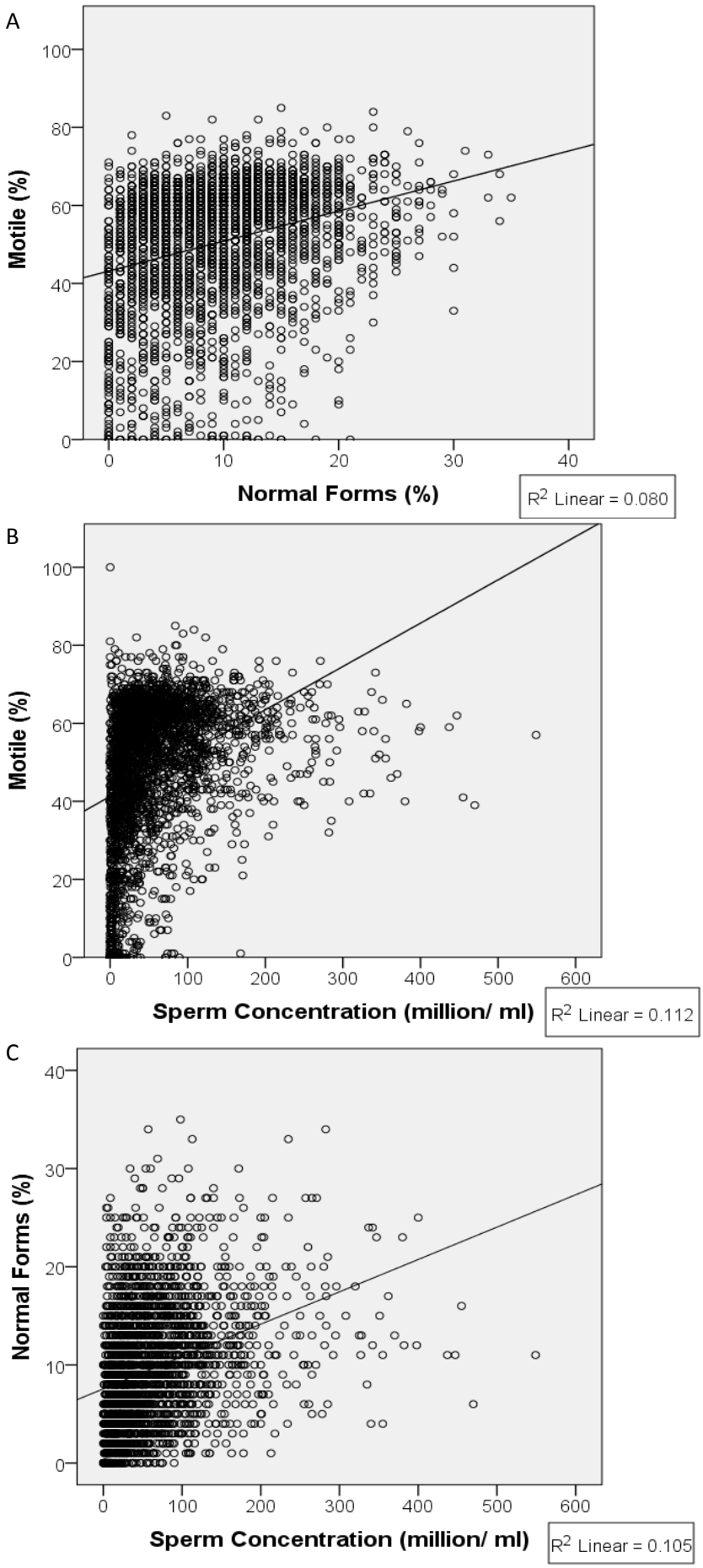


Figure 8-4 Scatter plots identifying correlations for Clinic A. 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C). N= 3239

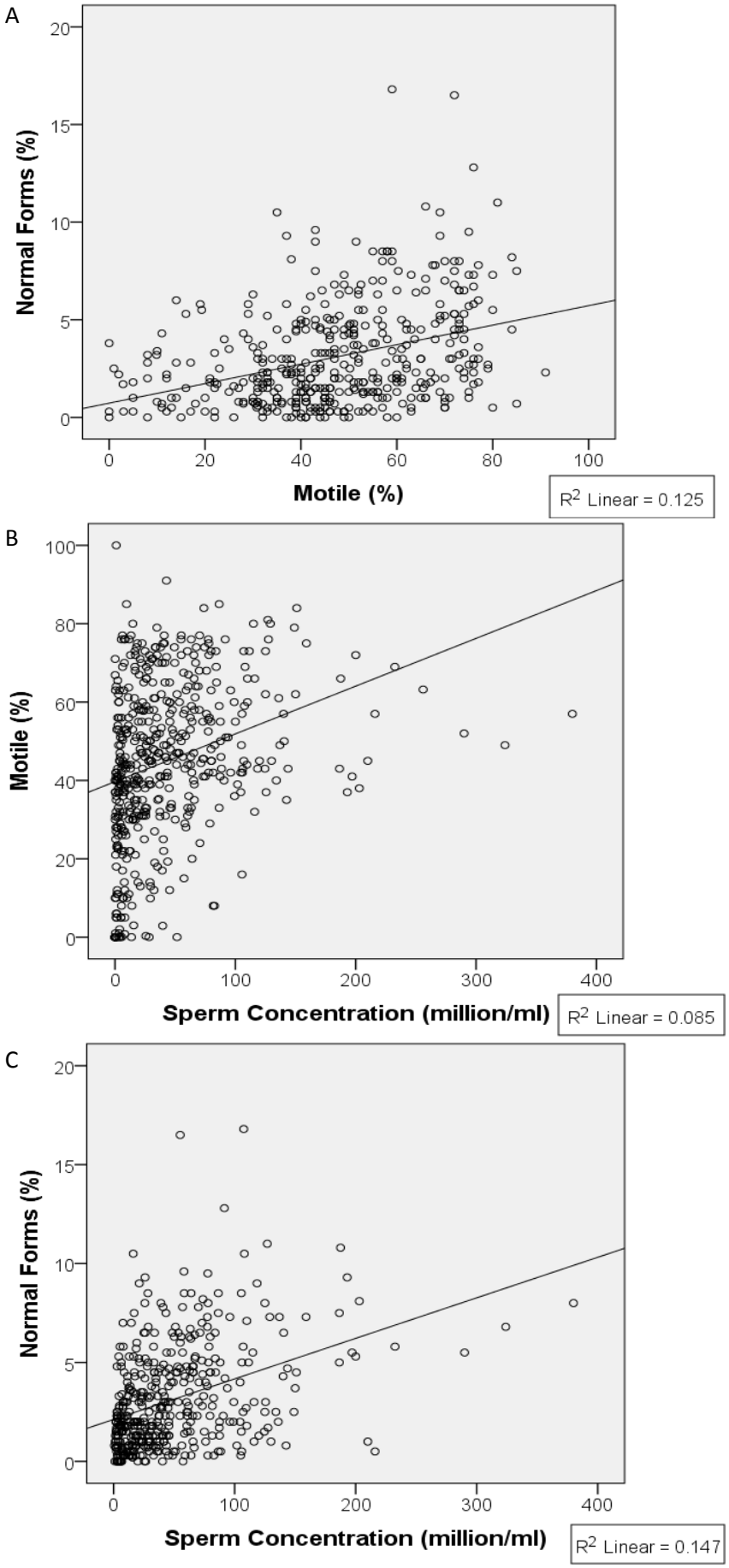


Figure 8-5 Scatter plots identifying correlations for Clinic B. 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C). N= 552

In an attempt to corroborate this, data from both clinics was further separated into two groups with a lower limit and upper limit: 1) semen with a concentration of less than, and including, 15 million/ml (WHO 2010 criteria) and 2) semen with a concentration of less than, and including, 100 million/ml. The same analysis as before was then conducted on the same pairs of variables within these two groups. When these same parameters (oligozoospermia, teratozoospermia and asthenozoospermia) were compared within their segregated groups the lower limit (less than 15 million/ml) once again identified a highly significant correlation ($P \leq 0.000$) for both clinic A and clinic B although, after visually comparing the parameters the scatter plots do not show a meaningful linear relationship (appendix). Linear regression analysis identifies that the total variance explained by the model is 12.2% with regards to clinic A and only 6% with regard to clinic B once again confirming a small correlation between the three parameters.

For the high concentration group, once again the correlations for both clinics are highly significant (clinic A; $p \leq 0.000$ - all parameters, clinic B; $p \leq 0.01$ - sperm concentration and motility, $p \leq 0.000$ motility and morphology and concentration and morphology). Linear regression analysis identified 6.5% variance is due to the model for clinic A and 11.4% for clinic B. This data highlights the variability between patients with the total percentage of variance explained by the model being greatest in the low concentration group for clinic A (12.2% vs 6% clinic B) whereas, the greatest variance explained by the model is greater in the high concentration group for clinic B (11.4% vs 6.5% clinic A). This data suggests that although a correlation does appear to exist between the three parameters it is only minimal identifying that each factor can be assessed independently and is not reliant on the other two parameters. This data suggests therapeutic advancements in motility are not restrained by the concentration and quantity of morphologically normal sperm.

As mentioned previously Publicover and Barratt (2011) identified that to increase the likelihood of a successful conception the ideal concentration of motile cells is ≥2 million/ml (Publicover and Barratt, 2011). Below this value small changes in the concentration of motile cells can have a noticeable effect on the chance of conception making it an ideal target area for motility enhancers. Using the data collected from the two centres it was possible to identify patients who, according to the graph from Publicover and Barratt (2011) (figure 8-1), would be suitable for and may benefit from *in vitro* drug treatment to increase motility. Data from clinic A highlighted 13% of patients (n=3015, azoospermic patients excluded) having a concentration of motile cells ≤2 million per ml with clinic B exhibiting 22% of patients (n=494, azoospermic patients excluded), under the same category, with the potential of benefiting from motility stimulants.

Data collected previously in this study (chapter 6) identified a potential therapeutic drug treatment (Trequinsin Hydrochloride) that can increase motility *in vitro*. This compound was capable of increasing total motility and specifically progressive motility by approximately 20%. Given this knowledge it is possible to predict that in patients with poor/low motility you would also see a 20% increase in motility of cells treated with this compound. Using guidelines from Ninewells Assisted Conception Unit it is possible to calculate the percentage of patients from both clinics who, after treatment with motility enhancing drugs, could be eligible for less invasive treatment options than currently used. Patients with concentrations of motile cells ≤1 million/ml would normally be treated with ICSI. The data for clinic A identified 1% (n=3015) whereas clinic B identified 3% (n=494) of patients after treatment with motility enhancing drugs, that could be eligible for IVF before the more invasive treatment of ICSI. To be eligible for IUI patients need to have a concentration of motile cells ≥5 million/ml, using this value as a guideline; it was possible to calculate that 3% of patients from clinic A

(n=3015) and 19% of patients from clinic B (n=494), after treatment, would be eligible for IUI instead of IVF.

Looking at sub-fertile patients (below WHO normal semen parameters) it was possible to generate a cumulative frequency curve identifying the percentage of patients from both clinic A and clinic B independently that have the potential of benefiting from treatment with motility stimulants (figure 8-6A). Looking at the curves from both clinics it is clear to see that they both follow a similar pattern indicating that the sub-fertile patients have similar profiles regardless of the spatio-temporal differences in data collection (figure 8-6A). Because the patient profiles follow a similar trend the data from the two clinics was combined to produce a single curve (figure 8-6B). In this analysis we are looking specifically at the potential of improving motility of patients who have a concentration of $\leq$2 million/ml motile cells and using the single curve it is possible to identify that approximately 60% of the sub-fertile patients from the two clinics combined fit this profile (figure 8-6B indicated by red line).

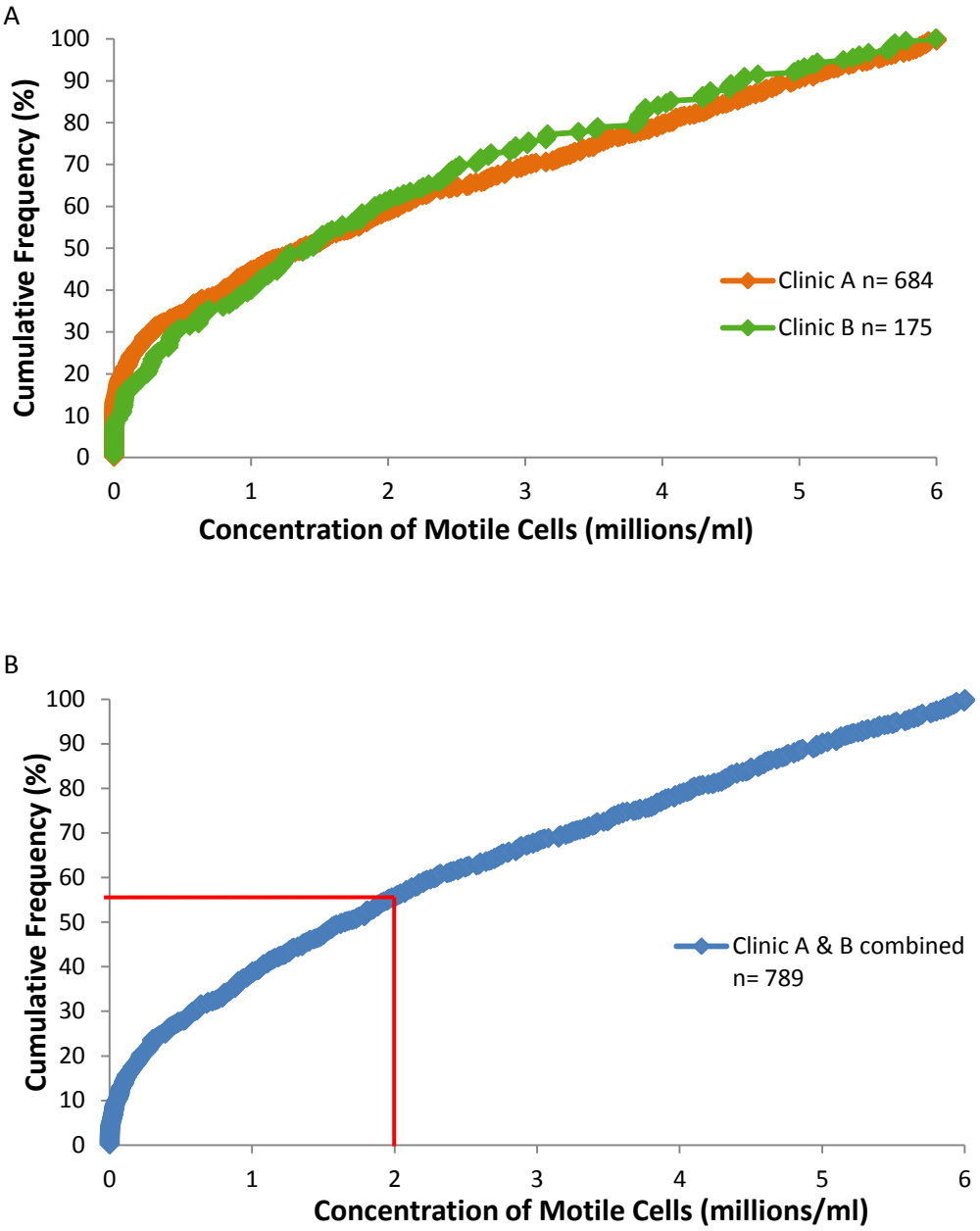


Figure 8-6 Cumulative frequency graph depicting the percentage of sub-fertile patients from clinic A and B independently (azoospermic patients included) (A) and combined (azoospermic patients excluded) (B). These graphs highlight the percentage of patients with below WHO normal values for concentration of motile cells. Using the graphs it is possible to determine the percentage of patients who could benefit from motility enhancing stimulants (red line).

8.4 Discussion

Motility dysfunction is major problem with regard to male infertility (Hull et al., 1985) with previous studies identifying isolated asthenozoospermia as the most frequent semen abnormality (Milardi et al., 2012). Similarly to Curi *et al* (2003) these dataset identified a higher percentage of patients with motility dysfunction in combination with factors such as poor morphology and/or low concentration than patients presenting with solely poor motility highlighting that novel treatments to improve motility could have a beneficial effect not only for couples with isolated motility problems but also with combined factors (Curi et al. 2003). This is further supported by this dataset and the findings of Macleod and Gold (1951) that sperm concentration contributes to sperm motility and this study further identifies sperm morphology as a contributing factor (Macleod and Gold, 1951b). The main aim of this study was to identify the sub-set of patients who would benefit clinically from treatments that specifically enhance motility. Combining the data from the two clinics 15% (n=3509) of patients could benefit from motility stimulants, according to the graph by Publicover and Barratt (2011) (Publicover and Barratt, 2011). By looking specifically at the sub-fertile patients (Below WHO normal concentrations of motile cells) it is possible to identify that almost 55% of these patients would be eligible for treatment with motility stimulants (excluding the azoospermic patients). This highlights the potential clinical benefits of successful treatment with motility enhancing stimulants.

The ability to increase success rates after IUI and make IUI more accessible to more patients through the use of motility stimulants would decrease the financial (Van Voorhis et al., 2001) and emotional burden on patients and healthcare providers. The proposed motility stimulants could significantly increase the number of progressively motile cells produced meaning that men who previously had below the threshold number of motile cells for IUI (between $1x10^6$-$3x10^6$ unprocessed sample) could now

be eligible for treatment (Tournaye and Cohlen, 2012, Tournaye, 2012, Van Voorhis et al., 2001). However, this data is based on semen analysis meaning it is not possible to guarantee that these samples would produce a minimum of $0.8x10^6$ total motile cells post preparation as required for IUI (Tournaye, 2012). Combining data from both Clinic A (1.8%) and Clinic B (1.2%) it has been possible to predict that 1.3% of patients, after treatment with motility enhancing drugs, could be eligible for IVF before the more invasive treatment of ICSI. Similarly it is possible to predict that a combined total of 5% (n=3509) of patients could now be considered for IUI as a first line treatment instead of IVF (Clinic A 2.6% n=3015, Clinic B 19.2% n=494). From this study it is only possible to predict the percentage of patients who previously were not eligible for IUI but after treatment with motility stimulants could become eligible. It is not possible to predict the potential beneficial effects motility stimulants could have on conception rates after treatment through IUI. However, there is good evidence that IUI is a cost effective treatment option that is less invasive than IVF and ICSI (Tournaye, 2012, Van Voorhis et al., 2001, Comhaire et al., 1995). With the discovery of novel compounds that enhance motility the success rates for fertilisation through IUI should increase making it a more viable option for more couples as an initial therapy for couples with male factor infertility.

There are a number of assumptions made from this data to be able to come to the conclusions drawn in this study. It is important to remember that even through improvement of motility you may not improve conception rates. There are a number of factors that contribute to a successful fertilisation that are not accounted for in this study including DNA abnormalities, inability to capacitate at the correct time, premature acrosome reaction and problems with phospholipase C Zeta activation to name but a few (Amdani et al., 2013, Donnelly, 2000).

CHAPTER 9

Conclusion and Future work

The primary aims of this thesis were to: (1) identify a sub-group of spermatozoa from donor samples that can be used, in research, as a representative sample for patient samples, (2) develop, and (3) implement a protocol for screening compounds, identified from a Flexstation screen, that have the possibility of enhancing motility through increasing $[Ca^{2+}]_i$ in sperm and finally, (4) establish the incidence of asthenozoospermia in patients who have previously undergone fertility treatment to identify patients who would benefit from therapeutic advancements in this area.

Key findings and future work

By establishing the optimum conditions *in vitro* (chapter 4) it was possible to develop a robust system for screening of motility enhancing compounds. Six compounds identified from the Chemogenomics library and fourteen compounds from the UoD DDU ion channel library were then tested using the standard operating procedure developed in chapter 4. The majority of these compounds were found to have no beneficial effects on sperm motility posing questions as to why these compounds increase $[Ca^{2+}]_i$ in sperm. To be able to further examine the effects of these compounds studies need to be conducted examining vitality and presence of the acrosome. With respect to chapter 5, the data highlights the importance of one compound identified from the Chemogenomics library; Trequinsin Hydrochloride. This data identified that Trequinsin increases $[Ca^{2+}]_i$ resulting in a significant increase in motility when measured by CASA and Kremer mucus penetration assays under non-capacitating conditions. This study, through the use of patch clamping, identifies CatSper activation by this PDEi suggesting that CatSper is an unidentified target of PDEi's in human sperm cells.

Trequinsin hydrochloride, after patch clamping, identified changes in human sperm potassium currents (hKSper). hKSper have been shown to originate in the sperm flagellum and have been found to support CatSper activity (Mannowetz et al., 2013). The human sperm principle potassium channel has been identified as Slo1 and is dependent on intracellular Ca^{2+} concentrations (Mannowetz et al., 2013). Lishko *et al* and Strunker *et al* independently discovered that CatSper is activated by progesterone by shifting Catsper activation to more hyperpolarised membrane potentials, increasing intracellular Ca^{2+} resulting in hyperactivated motility (Lishko et al., 2011, Strunker et al., 2011, Lishko et al., 2012a). Activation of CatSper is vital for fertilisation and since potassium channels are well known to regulate membrane potentials Lishko *et al* proposed their role in capacitation and specifically supporting CatSper induced hyperactivation (Navarro et al., 2007, Mannowetz et al., 2013). Slo1 has been found to be inhibited in a dose dependent manner by micromolar concentrations of progesterone which in an *in vivo* environment suggests that once the spermatozoa get in close proximity to the oocyte the hKSper channel will become blocked by increasing concentrations of progesterone, leading to membrane depolarization. This shift in membrane potential activates CatSper channels allowing for an influx of Ca^{2+} which in turn elevates $[Ca^{2+}]_{i.}$ This triggers Ca^{2+} dependent processes such as hyperactivated motility enabling spermatozoa to become capable of fertilisation (Mannowetz et al., 2013). This study proposes Trequinsin Hydrochloride functions in a similar manner to progesterone acting to inhibit Slo1 triggering membrane depolarisation thereby supporting full activation of CatSper. These events trigger hyperactivation allowing for penetration of the oocyte vestements and successful fertilisation.

The improvement in sperm motility attributed to treatment with B1 (chapter 7) were less pronounced than those seen in response to Trequinsin (chapter 6) with significant increases only visible when analysed by the Kremer mucus penetration assay. This

suggests that although it is possible B1 is activating CatSper it may not be an effective activator of sperm motility. B1 may only function via the CatSper channel which would allow for changes in flagella pattern to strongly enhance sperm penetration through viscous media but may not be capable of triggering Ca^{2+} release from internal stores preventing inducement of hyperactivation (Alasmari et al., 2013b). Thus it could be speculated that addition of this compound to spermatozoa from a therapeutic view point would not be wholly beneficial. If this compound were to be used in IUI procedures it appears it would be successful in aiding sperm penetration through cervical mucus but would not affect the sperms ability to, on reaching the oocyte, successfully penetrate the oocyte vestements to complete fertilisation.

One key issue that has been highlighted in this study is that use of 1% DMSO induces a premature acrosome reaction in cells treated with this compound for a long period of time (chapter 7). This is a similar problem to that found with use of pentoxifylline treatment previously (Tardif et al., 2014). One solution to this problem could be to examine the effects on motility of sperm preparations with a limited incubation with the motility enhancing compound which is then washed off. The motility of these sperm can then be examined after the wash to see if the motility enhancement is sustained reducing the risk of a premature acrosome reaction.

Whilst the work in this study has highlighted two potential compounds that successfully increase sperm motility; 1) Trequinsin hydrochloride (commercially available) and 2) B1 (previously unknown compound) these compounds have only been examined a limited number of times on both donor and patient samples. To categorically confirm their potential for increasing motility further studies are required. Furthermore, this work would need to be expanded on in order to confirm the proposed models in this study. Through the use of activators, inhibitors and/ or patch clamp studies experiments

need to be conducted to confirm that Trequinsin and B1 activate CatSper specifically and to investigate their effects on the human sperm potassium channel Slo1. These compounds also warrant closer examination to evaluate any unwanted effects such as; premature acrosome reaction or adverse effects caused by long incubations with the compounds. These compounds were identified as increasing $[Ca^{2+}]_i$ through a Flexstation Assay Screen to ensure this response is genuinely repeatable it would be beneficial to conduct fluorimetric population measurements on treated samples in parallel with motility analysis. To further clarify whether these compounds are activating CatSper alone or have any currently unidentified effects on Ca^{2+} stores the use of single cell imaging of samples would enable identification of Ca^{2+} store mobilisation as a result of treatment with these compounds. It would also be beneficial to conduct single cell Ca^{2+} imaging on sperm treated by each of the compounds to allow for exact identification of the increase in $[Ca^{2+}]_i$ seen as a result of drug stimulation.

This study has helped to develop a standard operating procedure for efficiently and reliably analysing the motility effects of sperm treated with compounds identified from the Flexstation screen. The results identified that in the future it would be beneficial to only analyse the effects of potential compounds on capacitated sperm. However, it is beneficial to analyse the effects on both the 40% fraction and 80% fraction from donor sperm. To ensure that the positive or negative effects on sperm are a true result of treatment with the compounds, in the future, the effect of leukocytes especially in the 40% fraction needs to be accounted for.

Male factor infertility is the cause of sub-fertility in 50% of cases with sperm dysfunction being the single most common factor responsible for male infertility (Hull et al., 1985, Thonneau et al., 1991). ART is currently the main way of treating male factor infertility but it is expensive and invasive (Comhaire et al., 1995, Van Voorhis et

al., 2001). The ability to treat more patients with IUI as a first line treatment for male factor infertility, through the use of motility stimulants, would make fertility treatment more accessible to couples worldwide who are not eligible for state funded treatments. This study identifies for the first time that through the use of motility enhancers it may be possible to treat a further 5% (chapter 8) of patients attending two independent fertility clinics with IUI before the more invasive procedure of IVF. It is also conceivable that these stimulants could not only increase the access to IUI (through making more patients eligible for treatment) but could potentially improve pregnancy rates within this treatment. This would make IUI a cost-effective, sustainable treatment option. This study has highlighted the percentage of sub-fertile patients from two independent fertility clinics. Through the use of a cumulus frequency curve it is possible to identify the percentage of patients, according to their concentration of motile cells, which could benefit from potential novel treatments. The similarity in the trend exhibited by the sub-fertile patients from the two independent clinics (chapter 8) suggests that this graph could be used in the future as a guideline for identifying target groups of patients for future studies.

References

ABU-HEIJA, A. T., FLEMING, R., JAMIESON, M. E., YATES, R. W. S. & COUTTS, J. R. T. 1996. The Effect of Sperm-Mucus Interaction Test on the Outcome of in vitro Fertilization and Ovulation Induction Combined with Intrauterin Insemination. *Journal of Obstetrics and Gynaecology Research,* 22**,** 229-233.

ADAMS, R. H., PORRAS, A., ALONSO, G., JONES, M., VINTERSTEN, K., PANELLI, S., VALLADARES, A., PEREZ, L., KLEIN, R. & NEBREDA, A. R. 2000. Essential Role of p38± MAP Kinase in Placental but Not Embryonic Cardiovascular Development. *Molecular cell,* 6**,** 109-116.

AGARWAL, A. & SAID, T. 2011. Interpretation of Basic Semen Analysis and Advanced Semen Testing. *In:* SABANEGH, E. S. (ed.) *Male Infertility.* Humana Press.

AGIRREGOITIA, E., CARRACEDO, A., SUBIRÁN, N., VALDIVIA, A., AGIRREGOITIA, N., PERALTA, L., VELASCO, G. & IRAZUSTA, J. 2010a. The CB2 cannabinoid receptor regulates human sperm cell motility. *Fertility and sterility,* 93**,** 1378-1387.

AGIRREGOITIA, E., CARRACEDO, A., SUBIRÁN, N., VALDIVIA, A., AGIRREGOITIA, N., PERALTA, L., VELASCO, G. & IRAZUSTA, J. 2010b. The CB(2) cannabinoid receptor regulates human sperm cell motility. *Fertility and sterility,* 93**,** 1378-87.

AITKEN, R. J. 2006. Sperm function tests and fertility. *International Journal of Andrology,* 29**,** 69-75.

AITKEN, R. J., BOWIE, H., BUCKINGHAM, D., HARKISS, D., RICHARDSON, D. W. & WEST, K. M. 1992. Sperm Penetration into a Hyaluronic Acid Polymer as a Means of Monitoring Functional Competence. *Journal of Andrology,* 13**,** 44-54.

AITKEN, R. J., BUCKINGHAM, D. W. & FANG, H. G. 1993. Analysis of the Responses of Human Spermatozoa to A23187 Employing a Novel Technique for Assessing the Acrosome Reaction. *Journal of Andrology,* 14**,** 132-141.

ALASMARI, W. 2013. *Regulation of calcium signalling for flagellar activity in human sperm and its significance in male fertility.* phD, University of Dundee.

ALASMARI, W., BARRATT, C. L. R., PUBLICOVER, S. J., WHALLEY, K. M., FOSTER, E., KAY, V., MARTINS DA SILVA, S. & OXENHAM, S. K. 2013a. The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Human reproduction (Oxford, England),* 28**,** 866-876.

ALASMARI, W., COSTELLO, S., CORREIA, J., OXENHAM, S. K., MORRIS, J., FERNANDES, L., RAMALHO-SANTOS, J., KIRKMAN-BROWN, J., MICHELANGELI, F., PUBLICOVER, S. & BARRATT, C. L. R. 2013b. Ca2+ Signals Generated by CatSper and Ca2+ Stores Regulate Different Behaviors in Human Sperm. *Journal of Biological Chemistry,* 288**,** 6248-6258.

ALMOG, T., LAZAR, S., REISS, N., ETKOVITZ, N., MILCH, E., RAHAMIM, N., DOBKIN-BEKMAN, M., ROTEM, R., KALINA, M., RAMON, J., RAZIEL, A., BRIETBART, H., SEGER, R. & NAOR, Z. 2008. Identification of Extracellular Signal-regulated Kinase 1/2 and p38 MAPK as Regulators of Human Sperm Motility and Acrosome Reaction and as Predictors of Poor Spermatozoan Quality. *Journal of Biological Chemistry,* 283**,** 14479-14489.

ALMOG, T. & NAOR, Z. 2008. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. *Molecular and Cellular Endocrinology,* 282**,** 39-44.

ALMOG, T. & NAOR, Z. 2010. The role of Mitogen activated protein kinase (MAPK) in sperm functions. *Molecular and Cellular Endocrinology,* 314**,** 239-243.

ALSENZ, J. & KANSY, M. 2007. High throughput solubility measurement in drug discovery and development. *Advanced drug delivery reviews,* 59**,** 546-67.

ALVAREZ, C. 2003. Biological variation of seminal parameters in healthy subjects. *Human Reproduction,* 18**,** 2082-2088.

AMOAKO, A. A., GEBEH, A. K., MARCZYLO, E. L., WILLETS, J. M., ELSON, J., MARCZYLO, T. H. & KONJE, J. C. 2013a. Impact of reference gene selection for type 2 cannabinoid receptor gene expression studies in human spermatozoa. *Andrologia,* 45**,** 278-284.

AMOAKO, A. A., MARCZYLO, T. H., LAM, P. M. W., WILLETS, J. M., DERRY, A., ELSON, J. & KONJE, J. C. 2010. Quantitative analysis of anandamide and related acylethanolamides

in human seminal plasma by ultra performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B,* 878**,** 3231-3237.

AMOAKO, A. A., MARCZYLO, T. H., MARCZYLO, E. L., ELSON, J., WILLETS, J. M., TAYLOR, A. H. & KONJE, J. C. 2013b. Anandamide modulates human sperm motility: implications for men with asthenozoospermia and oligoasthenoteratozoospermia. *Human Reproduction,* 28**,** 2058-2066.

ANDERSON, J. E., FARR, S. L., JAMIESON, D. J., WARNER, L. & MACALUSO, M. 2009. Infertility services reported by men in the United States: national survey data. *Fertility and Sterility,* 91**,** 2466-2470.

APPELL, R. A. & EVANS, P. R. 1977. The effect of temperature on sperm motility and viability. *Fertility and sterility,* 28**,** 1329-32.

ASHIZAWA, K., HASHIMOTO, K., HIGASHIO, M. & TSUZUKI, Y. 1997. The Addition of Mitogen-Activated Protein Kinase and p34cdc2Kinase Substrate Peptides Inhibits the Flagellar Motility of Demembranated Fowl Spermatozoa. *Biochemical and Biophysical Research Communications,* 240**,** 116-121.

ASHTON, J. C. & GLASS, M. 2007. The Cannabinoid CB2 Receptor as a Target for Inflammation-Dependent Neurodegeneration. *Curr Neuropharmacol,* 5**,** 73-80.

AUSTIN, C. R. 1951. Observations on the penetration of the sperm in the mammalian egg. *Australian journal of scientific research. Ser. B: Biological sciences,* 4**,** 581-96.

AVIDAN, N., TAMARY, H., DGANY, O., CATTAN, D., PARIENTE, A., THULLIEZ, M., BOROT, N., MOATI, L., BARTHELME, A., SHALMON, L., KRASNOV, T., ASHER, E. B., OLENDER, T., KHEN, M., YANIV, I., ZAIZOV, R., SHALEV, H., DELAUNAY, J., FELLOUS, M., LANCET, D. & BECKMANN, J. S. 2003. CATSPER2, a human autosomal nonsyndromic male infertility gene. *European Journal of Human Genetics,* 11**,** 497-502.

BAJPAI, M. & DONCEL, G. F. 2003. Involvement of tyrosine kinase and cAMP-dependent kinase cross-talk in the regulation of human sperm motility. *Reproduction,* 126**,** 183-195.

BALDI, E., CASANO, R., FALSETTI, C., KRAUSZ, C., MAGGI, M. & FORTI, G. 1991. INTRACELLULAR CALCIUM ACCUMULATION AND RESPONSIVENESS TO PROGESTERONE IN CAPACITATING HUMAN SPERMATOZOA. *Journal of Andrology,* 12**,** 323-330.

BALDI, E., LUCONI, M., BONACCORSI, L. & FORTI, G. 1998. Nongenomic effects of progesterone on spermatozoa: Mechanisms of signal transduction and clinical implications. *Frontiers in Bioscience,* 3.

BARRATT, C. L. R. 2011. The mystery is solved-CatSper is the principal calcium channel activated by progesterone in human spermatozoa. *Asian Journal of Andrology,* 13**,** 351-352.

BARRATT, C. L. R., MANSELL, S., BEATON, C., TARDIF, S. & OXENHAM, S. K. 2011. Diagnostic tools in male infertility-the question of sperm dysfunction. *Asian Journal of Andrology,* 13**,** 53-58.

BARRATT, C. L. R., OSBORN, J. C., HARRISON, P. E., MONKS, N., DUNPHY, B. C., LENTON, E. A. & COOKE, I. D. 1989. THE HYPO-OSMOTIC SWELLING TEST AND THE SPERM MUCUS PENETRATION TEST IN DETERMINING FERTILIZATION OF THE HUMAN OOCYTE. *Human Reproduction,* 4**,** 430-434.

BARRATT, C. L. R. & PUBLICOVER, S. J. 2012. Sperm are promiscuous and CatSper is to blame. *Embo Journal,* 31.

BATTISTONE, M. A., DA ROS, V. G., SALICIONI, A. M., NAVARRETE, F. A., KRAPF, D., VISCONTI, P. E. & CUASNICÚ, P. S. 2013. Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. *Molecular human reproduction***,** gat033--gat033-.

BAVISTER, B. D. 2002. Early history of in vitro fertilization. *Reproduction,* 124**,** 181-196.

BEAVO, J. A. 1995. CYCLIC-NUCLEOTIDE PHOSPHODIESTERASES - FUNCTIONAL IMPLICATIONS OF MULTIPLE ISOFORMS. *Physiological Reviews,* 75**,** 725-748.

BERGER, T., MARRS, R. P. & MOYER, D. L. 1985. COMPARISON OF TECHNIQUES FOR SELECTION OF MOTILE SPERMATOZOA. *Fertility and Sterility,* 43**,** 268-273.

BERWICK, D. C. & HARVEY, K. 2011. LRRK2 signaling pathways: the key to unlocking neurodegeneration? *Trends in Cell Biology,* 21**,** 257-265.

BIOSCIENCES, T. 2013a. *EO 1428 Supplier | CAS 321351-00-2 | Tocris Bioscience | EO1428* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5402 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013b. *GP 1a Supplier | Tocris Bioscience | GP1a* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5469 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013c. *JX 401 Supplier | CAS 349087-34-9 | Tocris Bioscience | JX401* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5188 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013d. *Leelamine Supplier | CAS 1446-61-3 | Tocris Bioscience | Dehydroabietylamine | Lylamine hydrochloride* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=109462 [Accessed 5th March 2013].

BIOSCIENCES, T. 2013e. *PHA 665752 Supplier | CAS 477575-56-7 | Tocris Bioscience | PHA665752* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=88278 [Accessed 13th August 2013].

BIOSCIENCES, T. 2013f. *Trequinsin Supplier | CAS 78416-81-6 | Tocris Bioscience | HL-725* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=142512 [Accessed 13th August 2013].

BJORNDAHL, L., MORTIMER, D., BARRATT, C. L. R., CASTILLA, J. A., MENKVELD, R., KVIST, U., ALVAREZ, J. G. & HAUGEN, T. B. 2010. *A Practical Guide to Basic Laboratory Andrology,* United Kingdom, Cambridge University Press.

BOATMAN, E. & ROBBINS, R. S. 1991a. Bicarbonate : Carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biology of reproduction,* 44**,** 806-813.

BOATMAN, E. & ROBBINS, R. S. 1991b. Bicarbonate : Regulation and of Sperm Hyperactivated Acrosome. *Biology of reproduction,* 44**,** 806-813.

BOIVIN, J., BUNTING, L., COLLINS, J. A. & NYGREN, K. G. 2007. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Human Reproduction,* 22**,** 1506-1512.

BOTTARO, D. P., RUBIN, J. S., FALETTO, D. L., CHAN, A. M. L., KMIECIK, T. E., VANDEWOUDE, G. F. & AARONSON, S. A. 1991. IDENTIFICATION OF THE HEPATOCYTE GROWTH-FACTOR RECEPTOR AS THE C-MET PROTOONCOGENE PRODUCT. *Science,* 251**,** 802-804.

BRAYTON, C. F. 1986. Dimethyl sulfoxide (DMSO): a review. *The Cornell veterinarian,* 76**,** 61-90.

BREITBART, H. 2002. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Molecular and Cellular Endocrinology,* 187**,** 139-144.

BRENKER, C., GOODWIN, N., WEYAND, I., KASHIKAR, N. D., NARUSE, M., KRAHLING, M., MULLER, A., KAUPP, U. B. & STRUNKER, T. 2012. The CatSper channel: a polymodal chemosensor in human sperm. *Embo Journal,* 31**,** 1654-1665.

BREWIS, I. A., MORTON, I. E., MOHAMMAD, S. N., BROWES, C. E. & MOORE, H. D. M. 2000. Measurement of intracellular calcium concentration and plasma membrane potential in human spermatozoa using flow cytometry. *Journal of Andrology,* 21**,** 238-249.

BURKMAN, L. J. 1991. DISCRIMINATION BETWEEN NONHYPERACTIVATED AND CLASSICAL HYPERACTIVATED MOTILITY PATTERNS IN HUMAN SPERMATOZOA USING COMPUTERIZED ANALYSIS. *Fertility and Sterility,* 55**,** 363-371.

BUZADZIC, B., VUCETIC, M., JANKOVIC, A., STANCIC, A., KORAC, A., KORAC, B. & OTASEVIC, V. 2014. New insights into male (in)fertility: The importance of NO. *British Journal of Pharmacology***,** n/a-n/a.

CALANDRA, B., PORTIER, M., KERNÉIS, A., DELPECH, M., CARILLON, C., LE FUR, G., FERRARA, P. & SHIRE, D. 1999. Dual intracellular signaling pathways mediated by the human cannabinoid CB1 receptor. *European Journal of Pharmacology,* 374**,** 445-455.

CAMPANA, A., SAKKAS, D., STALBERG, A., BIANCHI, P. G., COMTE, I., PACHE, T. & WALKER, D. 1996. Intrauterine insemination: evaluation of the results according to the woman's

age, sperm quality, total sperm count per insemination and life table analysis. *Human Reproduction,* 11**,** 732-736.

CARLSON, A. E., BURNETT, L. A., DEL CAMINO, D., QUILL, T. A., HILLE, B., CHONG, J. A., MORAN, M. M. & BABCOCK, D. F. 2009. Pharmacological Targeting of Native CatSper Channels Reveals a Required Role in Maintenance of Sperm Hyperactivation. *PLoS ONE,* 4**,** e6844.

CARRERA, A., MOOS, J., NING, X. P., GERTON, G. L., TESARIK, J., KOPF, G. S. & MOSS, S. B. 1996. Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: Identification of a kinase anchor proteins as major substrates for tyrosine phosphorylation. *Developmental Biology,* 180**,** 284-296.

CASTILLA, J. A., ALVAREZ, C., AGUILAR, J., GONZÁLEZ-VAREA, C., GONZALVO, M. C. & MARTÍNEZ, L. 2006. Influence of analytical and biological variation on the clinical interpretation of seminal parameters. *Human reproduction (Oxford, England),* 21**,** 847-51.

CATIZONE, A., RICCI, G., DEL BRAVO, J. & GALDIERI, M. 2006. Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. *Journal of Endocrinology,* 189**,** 137-146.

CATTERALL, W. A. 2000. STRUCTURE AND REGULATION OF VOLTAGE-GATED Ca2+ CHANNELS. *Annual Review of Cell and Developmental Biology,* 16**,** 521-555.

CATTERALL, W. A. & FEW, A. P. 2008. Calcium Channel Regulation and Presynaptic Plasticity. *Neuron,* 59**,** 882-901.

CHANG, M. C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature,* 168**,** 697-8.

CHAPEAU, C. & GAGNON, D. C. 1987. Nitrocellulose and Polyvinyl Coatings Prevent Sperm Adhesion to Glass Without Affecting the Motility of Intact and Demembranated Human Spermatozoa. *Journal of Andrology,* 8**,** 34-40.

CHEMES, H. E., BRUGO OLMEDO, S., CARRERE, C., OSES, R., CARIZZA, C., LEISNER, M. & BLAQUIER, J. 1998. Ultrastructural pathology of the sperm flagellum: association between flagellar pathology and fertility prognosis in severely asthenozoospermic men. *Human Reproduction,* 13**,** 2521-2526.

CHEN, Y. Q., CANN, M. J., LITVIN, T. N., IOURGENKO, V., SINCLAIR, M. L., LEVIN, L. R. & BUCK, J. 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science,* 289**,** 625-628.

CHULAVATNATOL, M. & HAESUNGCHARERN, A. 1977. Stabilization of adenylate energy charge and its relation to human sperm motility. *Journal of Biological Chemistry,* 252**,** 8088-8091.

COMHAIRE, F., MILINGOS, S., LIAPI, A., GORDTS, S., CAMPO, R., DEPYPERE, H., DHONT, M. & SCHOONJANS, F. 1995. The effective cumulative pregnancy rate of different modes of treatment of male infertility. *Andrologia,* 27**,** 217-221.

COOPER, T. G., NOONAN, E., VON ECKARDSTEIN, S., AUGER, J., BAKER, H. W. G., BEHRE, H. M., HAUGEN, T. B., KRUGER, T., WANG, C., MBIZVO, M. T. & VOGELSONG, K. M. 2010. World Health Organization reference values for human semen characteristics. *Human Reproduction Update,* 16**,** 231-245.

COSKRAN, T. M., MORTON, D., MENNITI, F. S., ADAMOWICZ, W. O., KLEIMAN, R. J., RYAN, A. M., STRICK, C. A., SCHMIDT, C. J. & STEPHENSON, D. T. 2006. Immunohistochemical Localization of Phosphodiesterase 10A in Multiple Mammalian Species. *Journal of Histochemistry & Cytochemistry,* 54**,** 1205-1213.

COSTELLO, S., MICHELANGELI, F., NASH, K., LEFIEVRE, L., MORRIS, J., MACHADO-OLIVEIRA, G., BARRATT, C., KIRKMAN-BROWN, J. & PUBLICOVER, S. 2009. Ca2+-stores in sperm: their identities and functions. *Reproduction,* 138**,** 425-437.

CRAVATT, B. F. & LICHTMAN, A. H. 2002. The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chemistry and Physics of Lipids,* 121**,** 135-148.

CURI, S. M., ARIAGNO, J. I., CHENLO, P. H., MENDELUK, G. R., PUGLIESE, M. N., SARDI SEGOVIA, L. M., REPETTO, H. E. H. & BLANCO, A. M. 2003. Asthenozoospermia: analysis of a large population. *Archives of andrology,* 49**,** 343-9.

DAI, S., HALL, D. D. & HELL, J. W. 2009. Supramolecular Assemblies and Localized Regulation of Voltage-Gated Ion Channels. *Physiological Reviews,* 89**,** 411-452.

DARSZON, A., ACEVEDO, J. J., GALINDO, B. E., HERNÁNDEZ-GONZÁLEZ, E. O., NISHIGAKI, T., TREVIÑO, C. L., WOOD, C. & BELTRÁN, C. 2006. Sperm channel diversity and functional multiplicity. *Reproduction,* 131**,** 977-988.

DARSZON, A., GUERRERO, A., GALINDO, B. E., NISHIGAKI, T. & WOOD, C. D. 2008. Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility. *International Journal of Developmental Biology,* 52**,** 595-606.

DARSZON, A., NISHIGAKI, T., BELTRAN, C. & TREVIÑO, C. L. 2011. Calcium channels in the development, maturation, and function of spermatozoa. *Physiological reviews,* 91**,** 1305-55.

DASCAL, N. 2001. Ion-channel regulated by G proteins. *TRENDS in Edocrinology & Metabolism,* 12**,** 391-398.

DAVID, G., SERRES, C. & JOUANNET, P. 1981. Kinematics of human spermatozoa. *Gamete Research,* 4**,** 83-95.

DE JONGE, C. 2005. Biological basis for human capacitation. *Human Reproduction Update,* 11**,** 205-214.

DE LAMIRANDE, E. & GAGNON, C. 2002. The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. *Molecular human reproduction,* 8**,** 124-35.

DE LAMIRANDE, E. & O'FLAHERTY, C. 2008. Sperm activation: Role of reactive oxygen species and kinases. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1784**,** 106-115.

DELAMIRANDE, E., LECLERC, P. & GAGNON, C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular Human Reproduction,* 3**,** 175-194.

DEMOTT, R. P. & SUAREZ, S. S. 1992. Hyperactivated sperm progress in the mouse oviduct. *Biology of Reproduction,* 46**,** 779-785.

DEPUYDT, C. E., DE POTTER, C. R., ZALATA, A., BAEKELANDT, E., BOSMANS, E. & COMHAIRE, F. H. 1998. Levels of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in Seminal Plasma of Patients With Andrological Diseases. *Journal of Andrology,* 19**,** 175-182.

DEPUYDT, C. E., ZALATA, A., DE POTTER, C. R., VAN EMMELO, J. & COMHAIRE, F. H. 1996. Genetic regulation of gametogensis: The receptor encoded by the human C-MET oncogene is expressed in testicular tissue and on human spermatozoa. *Molecular Human Reproduction,* 2**,** 2-8.

DEPUYDT, C. E., ZALATA, A., FALMAGNE, J. B., BOSMANS, E. & COMHAIRE, F. H. 1997. Purification and characterization of hepatocyte growth factor (HGF) from human seminal plasma. *International Journal of Andrology,* 20**,** 306-314.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. 1992. ISOLATION AND STRUCTURE OF A BRAIN CONSTITUENT THAT BINDS TO THE CANNABINOID RECEPTOR. *Science,* 258**,** 1946-1949.

DONNELLY, E. T., LEWIS, S. E., MCNALLY, J. A. & THOMPSON, W. 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. *Fertility and sterility,* 70**,** 305-14.

DOUGLAS-HAMILTON, D. H., SMITH, N. G., KUSTER, C. E., VERMEIDEN, J. P. W. & ALTHOUSE, G. C. 2005a. Capillary-Loaded Particle Fluid Dynamics: Effect on Estimation of Sperm Concentration. *Journal of Andrology,* 26**,** 115-122.

DOUGLAS-HAMILTON, D. H., SMITH, N. G., KUSTER, C. E., VERMEIDEN, J. P. W. & ALTHOUSE, G. C. 2005b. Particle Distribution in Low-Volume Capillary-Loaded Chambers. *Journal of Andrology,* 26**,** 107-114.

EGGERT-KRUSE, W., GERHARD, I., TILGEN, W. & RUNNEBAUM, B. 1989. CLINICAL-SIGNIFICANCE OF CROSSED INVITRO SPERM-CERVICAL MUCUS PENETRATION TEST IN INFERTILITY INVESTIGATION. *Fertility and Sterility,* 52**,** 1032-1040.

ESCOFFIER, J., BOISSEAU, S., SERRES, C., CHEN, C.-C., KIM, D., STAMBOULIAN, S., SHIN, H.-S., CAMPBELL, K. P., DE WAARD, M. & ARNOULT, C. 2007. Expression, localization and functions in acrosome reaction and sperm motility of CaV3.1 and CaV3.2 channels in sperm cells: An evaluation from CaV3.1 and CaV3.2 deficient mice. *Journal of Cellular Physiology,* 212**,** 753-763.

ESFANDIARI, N., SALEH, R. A., BLAUT, A. P., SHARMA, R. K., NELSON, D. R., THOMAS, A. J., FALCONE, T. & AGARWAL, A. 2002. Effects of temperature on sperm motion characteristics and reactive oxygen species. *International journal of fertility and women's medicine,* 47**,** 227-33.

ESHRE, E. S. O. H. R. A. E. 2012. The world's number of IVF and ICSI babies has now reached a calculated total of 5 million.

ESPOSITO, G., JAISWAL, B. S., XIE, F., KRAJNC-FRANKEN, M. A. M., ROBBEN, T., STRIK, A. M., KUIL, C., PHILIPSEN, R. L. A., VAN DUIN, M., CONTI, M. & GOSSEN, J. A. 2004. Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 2993-2998.

FAWCETT, L., BAXENDALE, R., STACEY, P., MCGROUTHER, C., HARROW, I., SODERLING, S., HETMAN, J., BEAVO, J. A. & PHILLIPS, S. C. 2000. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proceedings of the National Academy of Sciences,* 97**,** 3702-3707.

FISCH, J. D., BEHR, B. & CONTI, M. 1998. Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. *Human Reproduction,* 13**,** 1248-1254.

FISHER, D. A., SMITH, J. F., PILLAR, J. S., ST. DENIS, S. H. & CHENG, J. B. 1998. Isolation and Characterization of PDE8A, a Novel Human cAMP-Specific Phosphodiesterase. *Biochemical and Biophysical Research Communications,* 246**,** 570-577.

FORD, W. C. L., REES, J. M., MCLAUGHLIN, E. A., LING, L. & HULL, M. G. R. 1994. Pentoxifylline acts synergistically with A23187 to increase the penetration of zona-free hamster oocytes by cryopreserved human spermatozoa. *International Journal of Andrology,* 17**,** 199-204.

FORSTER, M. S., SMITH, W. D., LEE, W. I., BERGER, R. E., KARP, L. E. & STENCHEVER, M. A. 1983. SELECTION OF HUMAN-SPERMATOZOA ACCORDING TO THEIR RELATIVE MOTILITY AND THEIR INTERACTION WITH ZONA-FREE HAMSTER EGGS. *Fertility and Sterility,* 40**,** 655-660.

FRANCAVILLA, F., BATTISTA, N., BARBONETTI, A., VASSALLO, M. R. C., RAPINO, C., ANTONANGELO, C., PASQUARIELLO, N., CATANZARO, G., BARBONI, B. & MACCARRONE, M. 2009. Characterization of the Endocannabinoid System in Human Spermatozoa and Involvement of Transient Receptor Potential Vanilloid 1 Receptor in Their Fertilizing Ability. *Endocrinology,* 150**,** 4692-4700.

FRASER, C. G. 2001. *Biological variation: from principles to practice*, Amer. Assoc. for Clinical Chemistry.

FRASER, L., BARRATT, C. L., CANALE, D., COOPER, T., DEJONGE, C., IRVINE, S., MORTIMER, D., OEHNINGER, S. & TESARIK, J. 1997. Consensus workshop on advanced diagnostic andrology techniques. ESHRE Andrology Special Interest Group. *Human Reproduction,* 12**,** 873-873.

FRASER, L. & GROUP, S. I. 1998. Guidelines on the application of CASA technology in the analysis of spermatozoa. 13**,** 142-145.

FRASER, L. R. 1977. Motility patterns in mouse spermatozoa before and after capacitation. *J Exp Zool,* 202**,** 439-44.

FRASER, L. R. 1998. Sperm capacitation and the acrosome reaction. *Human Reproduction,* 13.

GADELLA, B. M. & VISCONTI, P. E. 2006. Regulation of capacitation. *In:* BARRATT, C. L. R. & DE JONGE, C. J. (eds.). Cambridge: Cambridge University Press.

GARNIER-LHOMME, M., BYRNE, R. D., HOBDAY, T. M. C., GSCHMEISSNER, S., WOSCHOLSKI, R., POCCIA, D. L., DUFOURC, E. J. & LARIJANI, B. 2009. Nuclear Envelope Remnants: Fluid Membranes Enriched in STEROLS and Polyphosphoinositides. *Plos One,* 4.

GARRETT, C., LIU, D. Y., CLARKE, G. N., RUSHFORD, D. D. & BAKER, H. W. G. 2003. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. *Human Reproduction,* 18**,** 1643-1649.

GHALENO, L. R., VALOJERDI, M. R., CHEHRAZI, M. & SHARBATOGHLI, M. 2013. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. *Archives of Gynecology and Obstetrics***,** 1-8.

GLASS, M. & FELDER, C. C. 1997. Concurrent Stimulation of Cannabinoid CB1 and Dopamine D2 Receptors Augments cAMP Accumulation in Striatal Neurons: Evidence for a Gs Linkage to the CB1 Receptor. *The Journal of Neuroscience,* 17**,** 5327-5333.

GOODWIN, L. O., KARABINUS, D. S., PERGOLIZZI, R. G. & BENOFF, S. 2000. L-type voltage-dependent calcium channel α-1C subunit mRNA is present in ejaculated human spermatozoa. *Molecular Human Reproduction,* 6**,** 127-136.

GOVERDE, A. J., MCDONNELL, J., VERMEIDEN, J. P. W., SCHATS, R., RUTTEN, F. F. H. & SCHOEMAKER, J. 2000. Intrauterine insemination or in-vitro fertilisation in idiopathic subfertility and male subfertility: a randomised trial and cost-effectiveness analysis. *The Lancet,* 355**,** 13-18.

GUERIN, J. F., MATHIEU, C., LORNAGE, J., PINATEL, M. C. & BOULIEU, D. 1989. IMPROVEMENT OF SURVIVAL AND FERTILIZING-CAPACITY OF HUMAN-SPERMATOZOA IN AN IVF PROGRAM BY SELECTION ON DISCONTINUOUS PERCOLL GRADIENTS. *Human Reproduction,* 4**,** 798-804.

GUTKIND, J. S. 1998. The Pathways Connecting G Protein-coupled Receptors to the Nucleus through Divergent Mitogen-activated Protein Kinase Cascades. *Journal of Biological Chemistry,* 273**,** 1839-1842.

GUZICK, D. S., OVERSTREET, J. W., FACTOR -L ITVAK, P. A. M., BRAZIL, C. K., NAKAJIMA, S. T., COUTIFARIS, C., CARSON, S. A., CISNEROS, P., STEINKAMPF, M. P., HILL, J. A., XU, D. & VOGEL, D. L. 2001. Sperm morphology, Motility, and Concentration in Fertile and Infertile Men. *The New England journal of Medicine,* 345**,** 1388-1393.

HAMAMAH, S., MAGNOUX, E., ROYERE, D., BARTHELEMY, C., DACHEUX, J. L. & GATTI, J. L. 1996. Internal pH of human spermatozoa: effect of ions, human follicular fluid and progesterone. *Molecular human reproduction,* 2**,** 219-24.

HARGREAVE, T. B. & ELTON, R. A. 1983. Is conventional sperm analysis of any use? *British journal of urology,* 55**,** 774-9.

HARPER, C. V., BARRATT, C. L. R. & PUBLICOVER, S. J. 2004. Stimulation of Human Spermatozoa with Progesterone Gradients to Simulate Approach to the Oocyte: INDUCTION OF [Ca2+]i OSCILLATIONS AND CYCLICAL TRANSITIONS IN FLAGELLAR BEATING. *Journal of Biological Chemistry,* 279**,** 46315-46325.

HERNESS, E. A. & NAZ, R. K. 1999. Presence and Tyrosine Phosphorylation of c-met Receptor in Human Sperm. *Journal of Andrology,* 20**,** 640-647.

HESS, R. A. & RENATO DE FRANCA, L. 2008. Spermatogenesis and cycle of the seminiferous epithelium. *Advances in experimental medicine and biology,* 636**,** 1-15.

HEUCHEL, V., SCHWARTZ, D. & CZYGLIK, F. 1983. BETWEEN AND WITHIN SUBJECT CORRELATIONS AND VARIANCES FOR CERTAIN SEMEN CHARACTERISTICS IN FERTILE MEN. *Andrologia,* 15**,** 171-176.

HILDEBRAND, M. S., AVENARIUS, M. R., FELLOUS, M., ZHANG, Y., MEYER, N. C., AUER, J., SERRES, C., KAHRIZI, K., NAJMABADI, H., BECKMANN, J. S. & SMITH, R. J. H. 2010. Genetic male infertility and mutation of CATSPER ion channels. *European Journal of Human Genetics,* 18.

HO, H. 2001. Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction,* 122**,** 519-526.

HO, H.-C. & SUAREZ, S. S. 2001. An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca2+ Store Is Involved in Regulating Sperm Hyperactivated Motility. *Biology of Reproduction,* 65**,** 1606-1615.

HOOGEWIJS, M. K., DE VLIEGHER, S. P., GOVAERE, J. L., DE SCHAUWER, C., DE KRUIF, A. & VAN SOOM, A. 2012. Influence of counting chamber type on CASA outcomes of equine semen analysis. *Equine veterinary journal,* 44**,** 542-9.

HOWLETT, A. C. 2005. Cannabinoids. *In:* PERTWEE, R. (ed.) *Handbook of Experimental Pharmacology.* Heidelberg, Germany: Springer-Verlag.

HOWLETT, A. C., BARTH, F., BONNER, T. I., CABRAL, G., CASELLAS, P., DEVANE, W. A., FELDER, C. C., HERKENHAM, M., MACKIE, K., MARTIN, B. R., MECHOULAM, R. & PERTWEE, R. G. 2002. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacological Reviews,* 54**,** 161-202.

HULL, M. G. R., GLAZENER, C. M. A., KELLY, N. J., CONWAY, D. I., FOSTER, P. A., HINTON, R. A., COULSON, C., LAMBERT, P. A., WATT, E. M. & DESAI, K. M. 1985. Population study of causes, treatment, and outcome of infertility. *British Medical Journal,* 291**,** 1693-1697.

IKAWA, M., INOUE, N., BENHAM, A. M. & OKABE, M. 2010. Fertilization: a sperm's journey to and interaction with the oocyte. *Journal of Clinical Investigation,* 120**,** 984-994.

IMAI, H., SUZUKI, K., ISHIZAKA, K., ICHINOSE, S., OSHIMA, H., OKAYASU, I., EMOTO, K., UMEDA, M. & NAKAGAWA, Y. 2001. Failure of the Expression of Phospholipid Hydroperoxide Glutathione Peroxidase in the Spermatozoa of Human Infertile Males. *Biology of Reproduction,* 64**,** 674-683.

IRVINE, D. S. 1998. Epidemiology and aetiology of male infertility. *Human Reproduction,* 13**,** 33-44.

IVIC, A. 2002. Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Human Reproduction,* 17**,** 143-149.

JAISWAL, B. S. & MAJUMDER, G. C. 1996. Cyclic AMP phosphodiesterase: A regulator of forward motility initiation during epididymal sperm maturation. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire,* 74**,** 669-674.

JEDRZEJCZAK, P., TASZAREK-HAUKE, G., HAUKE, J., PAWELCZYK, L. & DULEBA, A. J. 2008. Prediction of spontaneous conception based on semen parameters. *International Journal of Andrology,* 31**,** 499-507.

JEYENDRAN, R. S., VAN DER VEN, H. H., PEREZ-PELAEZ, M., CRABO, B. G. & ZANEVELD, L. J. D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility,* 70**,** 219-228.

JOHNSON, L. L., KATZ, D. F. & OVERSTREET, J. W. 1981. The movement characteristics of rabbit spermatozoa before and after activation. *Gamete Research,* 4**,** 275-282.

JONES, E. L., MUDRAK, O. & ANDREI, O. 2010. Kinetics of human male pronuclear development in a heterologous ICSI model. *Journal of Assisted Reproduction and Genetics,*27, 277-283.

JØRGENSEN, N., ANDERSEN, A. G., EUSTACHE, F., IRVINE, D. S., SUOMINEN, J., PETERSEN, J. H., ANDERSEN, A. N., AUGER, J., CAWOOD, E. H., HORTE, A., JENSEN, T. K., JOUANNET, P., KEIDING, N., VIERULA, M., TOPPARI, J. & SKAKKEBAEK, N. E. 2001. Regional differences in semen quality in Europe. *Human reproduction (Oxford, England),* 16**,** 1012-9.

KARANDE, V. C., KORN, A., MORRIS, R., RAO, R., BALIN, M., RINEHART, J., DOHN, K. & GLEICHER, N. 1999. Prospective randomized trial comparing the outcome and cost of in vitro fertilization with that of a traditional treatment algorithm as first-line therapy for couples with infertility. *Fertility and Sterility,* 71**,** 468-475.

KATZ, D., OVERSTREET, J. & HANSON, F. 1980. A new quantitative test for sperm penetration into cervical mucus. *Fertility and Sterility,* 33**,** 179-186.

KAY, V. J., COUTTS, J. R. T. & ROBERTSON, L. 1993. PENTOXIFYLLINE STIMULATES HYPERACTIVATION IN HUMAN SPERMATOZOA. *Human Reproduction,* 8**,** 727-731.

KEEL, B. A. 2006. Within- and between-subject variation in semen parameters in infertile men and normal semen donors. *Fertility and Sterility,* 85**,** 128-134.

KIRICHOK, Y. & LISHKO, P. V. 2011. Rediscovering sperm ion channels with the patch-clamp technique. *Molecular Human Reproduction,* 17**,** 478-499.

KIRICHOK, Y., NAVARRO, B. & CLAPHAM, D. E. 2006. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature,* 439**,** 737-740.

KITAMURA, M., MATSUMIYA, K., NISHIMURA, K., YAMANAKA, M., MATSUMOTO, K. & OKUYAMA, A. 2000. Effect of hepatocyte growth factor on sperm motility. *American Journal of Reproductive Immunology,* 44**,** 193-196.

KRAEMER, M., FILLION, C., MARTIN-PONT, B. & AUGER, J. 1998. Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. *Human reproduction (Oxford, England),* 13**,** 611-9.

KRASZNAI, Z., KRASZNAI, Z. T., MORISAWA, M., BAZSÁNÉ, Z. K., HERNÁDI, Z., FAZEKAS, Z., TRÓN, L., GODA, K. & MÁRIÁN, T. 2006. Role of the Na+/Ca2+ exchanger in calcium homeostasis and human sperm motility regulation. *Cell motility and the cytoskeleton,* 63**,** 66-76.

KRAUSZ, C., BONACCORSI, L., MAGGIO, P., LUCONI, M., CRISCUOLI, L., FUZZI, B., PELLEGRINI, S., FORTI, G. & BALDI, E. 1996. Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Human reproduction (Oxford, England),* 11**,** 1661-7.

KREMER, J. 1965. A SIMPLE SPERM PENETRATION TEST. *International Journal of Fertility,* 10**,** 209-&.

LAIL-TRECKER, M., GULATI, R. & PELUSO, J. J. 1998. A role for hepatocyte growth factor scatter factor in regulating normal and neoplastic cells of reproductive tissues. *Journal of the Society for Gynecologic Investigation,* 5**,** 114-121.

LARSEN, L., SCHEIKE, T., JENSEN, T. K. & BONDE, J. P. 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. *Human …,* 15**,** 1562-1567.

LE LANNOU, D., GRIVEAU, J. F., LE PICHON, J. P. & QUERO, J. C. 1992. Effects of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. *Human reproduction (Oxford, England),* 7**,** 1417-21.

LEE, M. A. & STOREY, B. T. 1986. BICARBONATE IS ESSENTIAL FOR FERTILIZATION OF MOUSE EGGS - MOUSE SPERM REQUIRE IT TO UNDERGO THE ACROSOME REACTION. *Biology of Reproduction,* 34**,** 349-356.

LEFIEVRE, L., BEDU-ADDO, K., CONNER, S. J., MACHADO-OLIVEIRA, G. S. M., CHEN, Y., KIRKMAN-BROWN, J. C., AFNAN, M. A., PUBLICOVER, S. J., FORD, W. C. L. & BARRATT, C. L. R. 2007. Counting sperm does not add up any more: time for a new equation? *Reproduction,* 133**,** 675-684.

LEFIEVRE, L., DE LAMIRANDE, E. & GAGNON, C. 2002. Presence of cyclic nucleotide phosphodiesterases PDE1, existing as a stable complex with calmodulin, and PDE3A in human spermatozoa. *Biology of Reproduction,* 67**,** 423-430.

LEFIEVRE, L., DE LAMIRANDE, E. V. E. & GAGNON, C. 2000. The Cyclic GMP-Specific Phosphodiesterase Inhibitor, Sildenafil, Stimulates Human Sperm Motility and Capacitation but Not Acrosome Reaction. *Journal of Andrology,* 21**,** 929-937.

LEFIEVRE, L., NASH, K., MANSELL, S., COSTELLO, S., PUNT, E., CORREIA, J., MORRIS, J., KIRKMAN-BROWN, J., WILSON, S., BARRATT, C. & PUBLICOVER, S. 2012. 2-APB-potentiated channels amplify CatSper-induced Ca2+ signals in human sperm. *Biochemical Journal,* 448**,** 189-200.

LEJA 2005. The Segre Silberberg effect.

LEWIS, S. E. M. 2007. Is sperm evaluation useful in predicting human fertility? *Reproduction,* 134**,** 31-40.

LI, M. W. M., MRUK, D. D. & CHENG, C. Y. 2009. Mitogen-activated protein kinases in male reproductive function. *Trends in Molecular Medicine,* 15**,** 159-168.

LINDEMANN, C. B. & KANOUS, K. S. 1989. REGULATION OF MAMMALIAN SPERM MOTILITY. *Archives of Andrology,* 23**,** 1-22.

LISHKO, P. V., BOTCHKINA, I. L., FEDORENKO, A. & KIRICHOK, Y. 2010. Acid Extrusion from Human Spermatozoa Is Mediated by Flagellar Voltage-Gated Proton Channel. *Cell,* 140**,** 327-337.

LISHKO, P. V., BOTCHKINA, I. L. & KIRICHOK, Y. 2011. Progesterone activates the principal Ca2+ channel of human sperm. *Nature,* 471**,** 387-391.

LISHKO, P. V. & KIRICHOK, Y. 2010. The role of Hv1 and CatSper channels in sperm activation. *The Journal of Physiology,* 588**,** 4667-4672.

LISHKO, P. V., KIRICHOK, Y., REN, D., NAVARRO, B., CHUNG, J.-J. & CLAPHAM, D. E. 2012a. The Control of Male Fertility by Spermatozoan Ion Channels. *Annual Review of Physiology,* 74**,** 453-475.

LISHKO, P. V., KIRICHOK, Y., REN, D. J., NAVARRO, B., CHUNG, J. J. & CLAPHAM, D. E. 2012b. The Control of Male Fertility by Spermatozoan Ion Channels. *In:* JULIUS, D. & CLAPHAM, D. E. (eds.) *Annual Review of Physiology, Vol 74.* Palo Alto: Annual Reviews.

LIU, D. Y. & BAKER, H. W. G. 1988. THE PROPORTION OF HUMAN-SPERM WITH POOR MORPHOLOGY BUT NORMAL INTACT ACROSOMES DETECTED WITH PISUM-SATIVUM AGGLUTININ CORRELATES WITH FERTILIZATION INVITRO. *Fertility and Sterility,* 50**,** 288-293.

LIU, D. Y., LIU, M. L., CLARKE, G. N. & BAKER, H. W. G. 2007. Hyperactivation of capacitated human sperm correlates with the zona pellucida-induced acrosome reaction of zona pellucida-bound sperm. *Human Reproduction,* 22**,** 2632-2638.

LIÉVANO, A., SANTI, C. M., SERRANO, C. J., TREVIÑO, C. L., BELLVÉ, A. R., HERNÁNDEZ-CRUZ, A. & DARSZON, A. 1996. T-type Ca2+ channels and α1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Letters,* 388**,** 150-154.

LOPEZ-GONZALEZ, I., DE LA VEGA-BELTRAN, J. L., SANTI, C. M., FLORMAN, H. M., FELIX, R. & DARSZON, A. 2001. Calmodulin antagonists inhibit T-type Ca2+ currents in mouse spermatogenic cells and the zona pellucida-induced sperm acrosome reaction. *Developmental Biology,* 236**,** 210-219.

LU, Q. & AN, W. F. 2008. Impact of novel screening technologies on ion channel drug discovery. *Combinatorial Chemistry & High Throughput Screening,* 11**,** 185-194.

MACCARRONE, M. 2008. CB2 receptors in reproduction. *British Journal of Pharmacology,* 153**,** 189-198.

MACLEOD, J. & GOLD, R. Z. 1951a. The Male Factor in Fertility and Infertility. II. Spermatozoon Counts in 1000 Men of Known Fertility and in 1000 Cases of Infertile Marriage. *The Journal of urology,* 66**,** 436-49.

MACLEOD, J. & GOLD, R. Z. 1951b. The Male Factor in Fertility and Infertility. III. An Analysis of Motile Activity in the Spermatozoa of 1000 Fertile Men and 1000 Men in Infertile Marriage. *Fertility and sterility,* 2**,** 187-204.

MAHI, C. A. & YANAGIMACHI, R. 1978. Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. *Gamete Research,* 1**,** 101-109.

MAHMOUD, A. M., DEPOORTER, B., PIENS, N. & COMHAIRE, F. H. 1997. The performance of 10 different methods for the estimation of sperm concentration. *Fertility and sterility,* 68**,** 340-5.

MAKLER, A., MAKLER, E., ITZKOVITZ, J. & BRANDES, J. M. 1980. Factors affecting sperm motility. IV. Incubation of human semen with caffeine, kallikrein, and other metabolically active compounds. *Fertility and sterility,* 33**,** 624-30.

MALLIDIS, C., HOWARD, E. J. & BAKER, H. W. G. 1991. VARIATION OF SEMEN QUALITY IN NORMAL MEN. *International Journal of Andrology,* 14**,** 99-107.

MANNOWETZ, N., NAIDOO, N. M., CHOO, S.-A. S., SMITH, J. F., LISHKO, P. V. & ALDRICH, R. 2013. Slo1 is the principal potassium channel of human spermatozoa. *eLife,* 2.

MANSELL, S. A., PUBLICOVER, S. J., BARRATT, C. L. R. & WILSON, S. M. 2014. Patch clamp studies of human sperm under physiological ionic conditions reveal three functionally and pharmacologically distinct cation channels. *Molecular Human Reproduction*.

MARTINS DA SILVA, S. J., HOPE, A. G., GRAY, D. W. & BARRATT, C. L. R. 2012. Validation of a novel high throughput screening assay to assess calcium responses in human sperm. *Fertility and Sterility,* 98**,** S84-S85.

MARZO, V. D., PETROCELLIS, L. D. & BISOGNO, T. 2005. The Biosynthesis, Fate and Pharmacological Properties of Endocannabinoids. *In:* PERTWEE, R. (ed.) *Cannabinoids: Handbook of Experimental Pharmacology.* Germany: Springer- Verlag.

MARÍN-BRIGGILER, C. I., JHA, K. N., CHERTIHIN, O., BUFFONE, M. G., HERR, J. C., VAZQUEZ-LEVIN, M. H. & VISCONTI, P. E. 2005. Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation. *Journal of Cell Science,* 118**,** 2013-2022.

MATSON, P. & TARDIF, S. 2012. A preliminary search for alternatives to albumin as a medium supplement for the culture of human sperm. *Reproductive Biology,* 12**,** 329-331.

MAYR, L. M. & BOJANIC, D. 2009. Novel trends in high-throughput screening. *Current Opinion in Pharmacology,* 9**,** 580-588.

MCLESKEY, S. B., DOWDS, C., CARBALLADA, R., WHITE, R. R. & SALING, P. M. 1998. Molecules involved in mammalian sperm-egg interaction. *International Review of Cytology - a Survey of Cell Biology, Vol 177,* 177.

MIKI, K., QU, W., GOULDING, E. H., WILLIS, W. D., BUNCH, D. O., STRADER, L. F., PERREAULT, S. D., EDDY, E. M. & O'BRIEN, D. A. 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 16501-16506.

MILARDI, D., GRANDE, G., SACCHINI, D., ASTORRI, A. L., POMPA, G., GIAMPIETRO, A., DE MARINIS, L., PONTECORVI, A., SPAGNOLO, A. G. & MARANA, R. 2012. Male fertility and reduction in semen parameters: a single tertiary-care center experience. *International journal of endocrinology,* 2012**,** 649149-649149.

MILLER, J. L. 2006. Recent developments in focused library design: Targeting gene-families. *Current Topics in Medicinal Chemistry,* 6**,** 19-29.

MOK, N. Y. & BRENK, R. 2011. Mining the ChEMBL Database: An Efficient Chemoinformatics Workflow for Assembling an Ion Channel-Focused Screening Library. *Journal of Chemical Information and Modeling,* 51**,** 2449-2454.

MOOHAN, J. M. & LINDSAY, K. S. 1995. SPERMATOZOA SELECTED BY A DISCONTINUOUS PERCOLL DENSITY GRADIENT EXHIBIT BETTER MOTION CHARACTERISTICS, MORE HYPERACTIVATION, AND LONGER SURVIVAL THAN DIRECT SWIM-UP. *Fertility and Sterility,* 64**,** 160-165.

MORISAWA, M. 1994. CELL SIGNALING MECHANISMS FOR SPERM MOTILITY. *Zoological Science,* 11**,** 647-662.

MORTIMER, D. 2000a. Sperm Preparation Methods. *Journal of Andrology,* 21**,** 357-366.

MORTIMER, D., AITKEN, R. J., MORTIMER, S. T. & PACEY, A. A. 1995. Workshop report: clinical CASA--the quest for consensus. *Reproduction, Fertility and Development,* 7**,** 951-951.

MORTIMER, D., COURTOT, A. M., GIOVANGRANDI, Y., JEULIN, C. & DAVID, G. 1984. Human sperm motility after migration into, and incubation in, synthetic media. *Gamete Research,* 9**,** 131-144.

MORTIMER, D. & MORTIMER, S. T. 2013a. Computer-Aided Sperm Analysis (CASA) of Sperm Motility and Hyperactivation. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, D. & MORTIMER, S. T. 2013b. Maunal Methods for Sperm Motility Assessment. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, D. & MORTIMER, S. T. 2013c. Spermatogenesis. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, S. T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. *Human Reproduction Update,* 3**,** 403-439.

MORTIMER, S. T. 2000b. CASA — Practical Aspects Andrology Lab Corner.

MORTIMER, S. T. & MORTIMER, D. 1990. Kinematics of Human Spermatozoa Incubated Under Capacitating Conditions. *Journal of Andrology,* 11**,** 195-203.

MORTIMER, S. T., SCHOEVAERT, D., SWAN, M. A. & MORTIMER, D. 1997. Quantitative observations of flagellar motility of capacitating human spermatozoa. *Human Reproduction,* 12**,** 1006-1012.

MORTIMER, S. T. & SWAN, M. A. 1995. Variable kinematics of capacitating human spermatozoa. *Human Reproduction,* 10**,** 3178-3182.

MORTIMER, S. T. & SWAN, M. A. 1999. Effect of image sampling frequency on established and smoothing-independent kinematic values of capacitating human spermatozoa. *Human Reproduction,* 14**,** 997-1004.

MORTIMER, S. T., SWAN, M. A. & MORTIMER, D. 1998. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Human Reproduction,* 13**,** 2139-2146.

MUKAI, C. & OKUNO, M. 2004. Glycolysis Plays a Major Role for Adenosine Triphosphate Supplementation in Mouse Sperm Flagellar Movement. *Biology of Reproduction,* 71**,** 540-547.

NALDINI, L., WEIDNER, K. M., VIGNA, E., GAUDINO, G., BARDELLI, A., PONZETTO, C., NARSIMHAN, R. P., HARTMANN, G., ZARNEGAR, R., MICHALOPOULOS, G. K., BIRCHMEIER, W. & COMOGLIO, P. M. 1991. SCATTER FACTOR AND HEPATOCYTE GROWTH-FACTOR ARE INDISTINGUISHABLE LIGANDS FOR THE MET RECEPTOR. *Embo Journal,* 10**,** 2867-2878.

NAVARRO, B., KIRICHOK, Y. & CLAPHAM, D. E. 2007. KSper, a pH-sensitive K+ current that controls sperm membrane potential. *Proceedings of the National Academy of Sciences,* 104**,** 7688-7692.

NAZ, R. K. 1996. Protein tyrosine phosphorylation and signal transduction during capacitation-acrosome reaction and zona pellucida binding in human sperm. *Archives of Andrology,* 37**,** 47-55.

NAZ, R. K., AHMAD, K. & KUMAR, R. 1991. ROLE OF MEMBRANE PHOSPHOTYROSINE PROTEINS IN HUMAN SPERMATOZOAL FUNCTION. *Journal of Cell Science,* 99**,** 157-165.

NAZ, R. K., JOSEPH, A., LEE, Y., AHMAD, K. & BHARGAVA, M. M. 1994. Expression of scatter factor/hepatocyte growth factor is regionally correlated with the initiation of sperm motility in murine male genital tract: Is scatter factor/hepatocyte growth factor involved in initiation of sperm motility? *Molecular Reproduction and Development,* 38**,** 431-439.

NAZ, R. K. & RAJESH, P. B. 2004. Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reproductive biology and endocrinology : RB&E,* 2**,** 75.

NICE, N. I. F. H. A. C. E. 2013. Fertility:assessment and treatment for people with fertility problems. 2nd edition ed. London: National Collaborating Centre for Women's and Children's Health.

O'FLAHERTY, C., DE LAMIRANDE, E. & GAGNON, C. 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radical Biology and Medicine,* 41**,** 528-540.

OACONNELL, M. 2003. Differences in mitochondrial and nuclear DNA status of high-density and low-density sperm fractions after density centrifugation preparation*1. *Fertility and Sterility,* 79**,** 754-762.

OKAMOTO, Y., WANG, J., MORISHITA, J. & UEDA, N. 2007. Biosynthetic pathways of the endocannabinoid anandamide. *Chemistry & Biodiversity,* 4**,** 1842-1857.

OLSON, S., SUAREZ, S. & FAUCI, L. 2010. A Model of CatSper Channel Mediated Calcium Dynamics in Mammalian Spermatozoa. *Bulletin of Mathematical Biology,* 72**,** 1925-1946.

OLSON, S. D., FAUCI, L. J. & SUAREZ, S. S. 2011. Mathematical modeling of calcium signaling during sperm hyperactivation. *Molecular Human Reproduction,* 17**,** 500-510.

OMBELET, W., BOSMANS, E., JANSSEN, M., COX, A., VLASSELAER, J., GYSELAERS, W., VANDEPUT, H., GIELEN, J., POLLET, H., MAES, M., STEENO, O. & KRUGER, T. 1997. Semen parameters in a fertile versus subfertile population: A need for change in the interpretation of semen testing. *Human Reproduction,* 12**,** 987-993.

PACEY, A. A., DAVIES, N., WARREN, M. A., BARRATT, C. L. R. & COOKE, L. D. 1995. Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. *Human Reproduction,* 10**,** 2603-2609.

PARK, J.-Y., AHN, H.-J., GU, J.-G., LEE, K.-H., KIM, J.-S., KANG, H.-W. & LEE, J.-H. 2003. Molecular identification of Ca(2+)channels in human sperm. *Exp Mol Med,* 35**,** 285-292.

PEARSON, G., ROBINSON, F., BEERS GIBSON, T., XU, B.-E., KARANDIKAR, M., BERMAN, K. & COBB, M. H. 2001. Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. *Endocrine Reviews,* 22**,** 153-183.

PERTWEE, R. G. 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology & Therapeutics,* 74**,** 129-180.

PERTWEE, R. G. 2005. Pharmacological Actions of Cannabinoids. *In:* PERTWEE, R. (ed.) *Cannabinoids.* Springer Berlin Heidelberg.

POLAND, M. L., MOGHISSI, K. S., GIBLIN, P. T., AGER, J. W. & OLSON, J. M. 1985. VARIATION OF SEMEN MEASURES WITHIN NORMAL MEN. *Fertility and Sterility,* 44**,** 396-400.

PORRAS, A., ZULUAGA, S., BLACK, E., VALLADARES, A., ALVAREZ, A. M., AMBROSINO, C., BENITO, M. & NEBREDA, A. R. 2004. p38α Mitogen-activated Protein Kinase Sensitizes Cells to Apoptosis Induced by Different Stimuli. *Molecular Biology of the Cell,* 15**,** 922-933.

PUBLICOVER, S., HARPER, C. V. & BARRATT, C. 2007. [Ca2+]i signalling in sperm-making the most of what you've got. *Nature Cell Biology,* 9**,** 235-42.

PUBLICOVER, S. J. & BARRATT, C. L. R. 2011. Sperm motility: things are moving in the lab! *Molecular Human Reproduction,* 17**,** 453-456.

QI, H., MORAN, M. M., NAVARRO, B., CHONG, J. A., KRAPIVINSKY, G., KRAPIVINSKY, L., KIRICHOK, Y., RAMSEY, I. S., QUILL, T. A. & CLAPHAM, D. E. 2007. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proceedings of the National Academy of Sciences,* 104**,** 1219-1223.

RAMMLER, D. H. & ZAFFARONI, A. 1967. BIOLOGICAL IMPLICATIONS OF DMSO BASED ON A REVIEW OF ITS CHEMICAL PROPERTIES. *Annals of the New York Academy of Sciences,* 141**,** 13-23.

RAUPRICH, O., BERNS, E. & VOLLMANN, J. 2010. Who should pay for assisted reproductive techniques? Answers from patients, professionals and the general public in Germany. *Human reproduction (Oxford, England),* 25**,** 1225-33.

REN, D. & XIA, J. 2010. Calcium Signaling Through CatSper Channels in Mammalian Fertilization. *Physiology,* 25**,** 165-175.

REN, D. J., NAVARRO, B., PEREZ, G., JACKSON, A. C., HSU, S. F., SHI, Q., TILLY, J. L. & CLAPHAM, D. E. 2001. A sperm ion channel required for sperm motility and male fertility. *Nature,* 413**,** 603-609.

RICHTER, W., DETTMER, D. & GLANDER, H. J. 1999. Detection of mRNA transcripts of cyclic nucleotide phosphodiesterase subtypes in ejaculated human spermatozoa. *Molecular Human Reproduction,* 5**,** 732-736.

ROSSATO, M., ION POPA, F., FERIGO, M., CLARI, G. & FORESTA, C. 2005. Human Sperm Express Cannabinoid Receptor Cb1, the Activation of Which Inhibits Motility, Acrosome Reaction, and Mitochondrial Function. *Journal of Clinical Endocrinology & Metabolism,* 90**,** 984-991.

ROSSI, P., PEZZOTTI, R., CONTI, M. & GEREMIA, R. 1985. Cyclic nucleotide phosphodiesterases in somatic and germ cells of mouse seminiferous tubules. *Journal of Reproduction and Fertility,* 74**,** 317-327.

ROWE, P. C., FRANK. HARGREAVE, TIMOTHY. MELLOWS, HEATHER. 1993. *WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple,* Cambridge, Cambridge University Press.

SAAD, A. & GUERIN, J. F. 1992. Movement characteristics of human spermatozoa collected from different layers of a discontinuous Percoll gradient. *Andrologia,* 24**,** 149-153.

SAGARE-PATIL, V., GALVANKAR, M., SATIYA, M., BHANDARI, B., GUPTA, S. K. & MODI, D. 2012. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *International Journal of Andrology,* 35**,** 633-644.

SALICIONI, A. M., PLATT, M. D., WERTHEIMER, E. V., ARCELAY, E., ALLAIRE, A., SOSNIK, J. & VISCONTI, P. E. 2007. Signalling pathways involved in sperm capacitation. *Society of Reproduction and Fertility supplement,* 65**,** 245-59.

SANTI, C. M., MARTÍNEZ-LÓPEZ, P., DE LA VEGA-BELTRÁN, J. L., BUTLER, A., ALISIO, A., DARSZON, A. & SALKOFF, L. 2010. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS Letters,* 584**,** 1041-1046.

SCHMIDT, L., MÜNSTER, K. & HELM, P. 1995. Infertility and the seeking of infertility treatment in a representative population. *British journal of obstetrics and gynaecology,* 102**,** 978-84.

SCHREIBER, M., WEI, A., YUAN, A., GAUT, J., SAITO, M. & SALKOFF, L. 1998. Slo3, a Novel pH-sensitive K+ Channel from Mammalian Spermatocytes. *Journal of Biological Chemistry,* 273**,** 3509-3516.

SCHREIBER, M., YUAN, A. & SALKOFF, L. 1999. Transplantable sites confer calcium sensitivity to BK channels. *Nature Neuroscience,* 2**,** 416-421.

SCHUEL, H., BURKMAN, L. J., LIPPES, J., CRICKARD, K., MAHONY, M. C., GIUFFRIDA, A., PICONE, R. P. & MAKRIYANNIS, A. 2002. Evidence that anandamide-signaling regulates human sperm functions required for fertilization. *Molecular Reproduction and Development,* 63**,** 376-387.

SERRANO, C. J., TREVIÑO, C. L., FELIX, R. & DARSZON, A. 1999. Voltage-dependent Ca2+ channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. *FEBS Letters,* 462**,** 171-176.

SERVIN-VENCES, M. R., TATSU, Y., ANDO, H., GUERRERO, A., YUMOTO, N., DARSZON, A. & NISHIGAKI, T. 2012. A caged progesterone analog alters intracellular Ca2+ and flagellar bending in human sperm. *Reproduction,* 144**,** 101-109.

SI, Y. & OKUNO, M. 1995. Activation of mammalian sperm motility by regulation of microtubule sliding via cyclic adenosine 5'-monophosphate-dependent phosphorylation. *Biology of Reproduction,* 53**,** 1081-1087.

SIGNORELLI, J., DIAZ, E. S. & MORALES, P. 2012. Kinases, phosphatases and proteases during sperm capacitation. *Cell and Tissue Research,* 349**,** 765-782.

SINGH, J. P., BABCOCK, D. F. & LARDY, H. A. 1983. Motility activation, respiratory stimulation, and alteration of Ca2+ transport in bovine sperm treated with amine local anesthetics

and calcium transport antagonists. *Archives of Biochemistry and Biophysics,* 221**,** 291-303.

SODERLING, S. H. & BEAVO, J. A. 2000. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Current Opinion in Cell Biology,* 12**,** 174-179.

STONE, B. A., VARGYAS, J. M., RINGLER, G. E., STEIN, A. L. & MARRS, R. P. 1999. Determinants of the outcome of intrauterine insemination: Analysis of outcomes of 9963 consecutive cycles. *American Journal of Obstetrics and Gynecology,* 180**,** 1522-1534.

STRAIN, A. J. 1993. HEPATOCYTE GROWTH-FACTOR - ANOTHER UBIQUITOUS CYTOKINE. *Journal of Endocrinology,* 137**,** 1-5.

STRUNKER, T., GOODWIN, N., BRENKER, C., KASHIKAR, N. D., WEYAND, I., SEIFERT, R. & KAUPP, U. B. 2011. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature,* 471**,** 382-386.

SUAREZ, S. S. 2008. Control of hyperactivation in sperm. *Human Reproduction Update,* 14**,** 647-657.

SUAREZ, S. S., KATZ, D. F., OWEN, D. H., ANDREW, J. B. & POWELL, R. L. 1991. Evidence for the function of hyperactivated motility in sperm. *Biology of Reproduction,* 44**,** 375-381.

SUAREZ, S. S., VAROSI, S. M. & DAI, X. 1993. Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *Proceedings of the National Academy of Sciences,* 90**,** 4660-4664.

SUGIURA, T., KODAKA, T., KONDO, S., NAKANE, S., KONDO, H., WAKU, K., ISHIMA, Y., WATANABE, K. & YAMAMOTO, I. 1997. Is the Cannabinoid CB1 Receptor a 2-Arachidonoylglycerol Receptor? Structural Requirements for Triggering a Ca2+ Transient in NG108–15 Cells. *Journal of Biochemistry,* 122**,** 890-895.

SUGIURA, T. & WAKU, K. 2000. 2-Arachidonoylglycerol and the cannabinoid receptors. *Chemistry and Physics of Lipids,* 108**,** 89-106.

SUKCHAROEN, N., KEITH, J., IRVINE, D. S. & AITKEN, R. J. 1995. PREDICTING THE FERTILIZING POTENTIAL OF HUMAN SPERM SUSPENSIONS IN-VITRO - IMPORTANCE OF SPERM MORPHOLOGY AND LEUKOCYTE CONTAMINATION. *Fertility and Sterility,* 63.

SUKCHAROEN, N., KEITH, J., IRVINE, D. S. & AITKEN, R. J. 1996. Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Human Reproduction,* 11**,** 1030-1034.

TASH, J. S. 1990. Role of cAMP, calcium, and protein phosphorylation in sperm motility. *Controls of sperm motility: biological and clinical aspects.***,** 229-240.

TERSTAPPEN, G. C., RONCARATI, R., DUNLOP, J. & PERI, R. 2010. Screening technologies for ion channel drug discovery. *Future Medicinal Chemistry,* 2**,** 715-730.

TESARIK, J., MENDOZA, C. & CARRERAS, A. 1992. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertility and sterility,* 58**,** 1185-1190.

TESARIKI, J., THÉBAULT, A. & TESTART, J. 1992. Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. *Human Reproduction,* 7**,** 1257-1263.

TESAŘÍK, J., OLTRAS, C. M. & TESTART, J. 1990. Effect of the human cumulus oophorus on movement characteristics of human capacitated spermatozoa. *Journal of Reproduction and Fertility,* 88**,** 665-675.

THONNEAU, P., MARCHAND, S., TALLEC, A., FERIAL, M.-L., DUCOT, B., LANSAC, J., LOPES, P., TABASTE, J.-M. & SPIRA, A. 1991. Incidence and main causes of infertility in a resident population (1 850 000) of three French regions (1988–1989)*. *Human Reproduction,* 6**,** 811-816.

TOMLINSON, M., LEWIS, S. & MORROLL, D. 2013. Sperm quality and its relationship to natural and assisted conception: British Fertility Society Guidelines for practice. *Human Fertility,* 16**,** 175-193.

TOMLINSON, M., TURNER, J., POWELL, G. & SAKKAS, D. 2001. One-step disposable chambers for sperm concentration and motility assessment : how do they compare with the World Health Organization ' s recommended methods ? 16**,** 121-124.

TOMLINSON, M. J., KESSOPOULOU, E. & BARRATT, C. L. 1999. The diagnostic and prognostic value of traditional semen parameters. *Journal of Andrology,* 20**,** 588-593.

TOMLINSON, M. J., POOLEY, K., SIMPSON, T., NEWTON, T., HOPKISSON, J., JAYAPRAKASAN, K., JAYAPRAKASAN, R., NAEEM, A. & PRIDMORE, T. 2010. Validation of a novel computer-assisted sperm analysis (CASA) system using multitarget-tracking algorithms. *Fertility and sterility,* 93**,** 1911-20.

TOURNAYE, H. 2012. Male factor infertility and ART. *Asian journal of andrology,* 14**,** 103-8.

TOURNAYE, H. J. & COHLEN, B. J. 2012. Management of male-factor infertility. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 26**,** 769-775.

TSIEN, R. W., ELLINOR, P. T. & HORNE, W. A. 1991. MOLECULAR DIVERSITY OF VOLTAGE-DEPENDENT CA2+ CHANNELS. *Trends in Pharmacological Sciences,* 12**,** 349-&.

UEHARA, Y., MINOWA, O., MORI, C., SHIOTA, K., KUNO, J., NODA, T. & KITAMURA, N. 1995. PLACENTAL DEFECT AND EMBRYONIC LETHALITY IN MICE LACKING HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR. *Nature,* 373**,** 702-705.

ULLRICH, A. & SCHLESSINGER, J. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell,* 61**,** 203-212.

VAN VOORHIS, B. J., BARNETT, M., SPARKS, A. E., SYROP, C. H., ROSENTHAL, G. & DAWSON, J. 2001. Effect of the total motile sperm count on the efficacy and cost-effectiveness of intrauterine insemination and in vitro fertilization. *Fertility and sterility,* 75**,** 661-8.

VAN WEERT, J. M., REPPING, S., VAN VOORHIS, B. J., VAN DER VEEN, F., BOSSUYT, P. M. M. & MOL, B. W. J. 2004. Performance of the postwash total motile sperm count as a predictor of pregnancy at the time of intrauterine insemination: a meta-analysis. *Fertility and Sterility,* 82**,** 612-620.

VASTA, V., SONNENBURG, W. K., YAN, C., SODERLING, S. H., SHIMIZU-ALBERGINE, M. & BEAVO, J. A. 2005. Identification of a New Variant of PDE1A Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterase Expressed in Mouse Sperm. *Biology of Reproduction,* 73**,** 598-609.

VISCONTI, P. E. 2009. Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 667-8.

VISCONTI, P. E., BAILEY, J. L., MOORE, G. D., PAN, D., OLDS-CLARKE, P. & KOPF, G. S. 1995a. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development (Cambridge, England),* 121**,** 1129-37.

VISCONTI, P. E., JOHNSON, L. R., OYASKI, M., FORNES, M., MOSS, S. B., GERTON, G. L. & KOPF, G. S. 1997. Regulation, localization, and anchoring of protein kinase a subunits during mouse sperm capacitation. *Developmental Biology,* 192**,** 351-363.

VISCONTI, P. E., KRAPF, D., DE LA VEGA-BELTRÁN, J. L., ACEVEDO, J. J. & DARSZON, A. 2011. Ion channels, phosphorylation and mammalian sperm capacitation. *Asian journal of andrology,* 13**,** 395-405.

VISCONTI, P. E., MOORE, G. D., BAILEY, J. L., LECLERC, P., CONNORS, S. A., PAN, D. Y., OLDSCLARKE, P. & KOPF, G. S. 1995b. CAPACITATION OF MOUSE SPERMATOZOA .2. PROTEIN-TYROSINE PHOSPHORYLATION AND CAPACITATION ARE REGULATED BY A CAMP-DEPENDENT PATHWAY. *Development,* 121**,** 1139-1150.

VISCONTI, P. E., MOORE, G. D., BAILEY, J. L., LECLERC, P., CONNORS, S. A., PAN, D. Y., OLDSCLARKE, P. & KOPF, G. S. 1995c. Capacitation of Mouse Spermatozoa. 2. Protein-Tyrosine Phosphorylation and Capacitation are Regulated by a cAMP-dependent Pathway. *Development,* 121**,** 1139-1150.

VISCONTI, P. E., WESTBROOK, V. A., CHERTIHIN, O., DEMARCO, I., SLEIGHT, S. & DIEKMAN, A. B. 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *Journal of reproductive immunology,* 53**,** 133-50.

VISSER, L., WESTERVELD, G. H., XIE, F., VAN DAALEN, S. K. M., VAN DER VEEN, F., LOMBARDI, M. P. & REPPING, S. 2011. A comprehensive gene mutation screen in men with asthenozoospermia. *Fertility and Sterility,* 95**,** 1020-1024.e9.

WALENSKY, L. D. & SNYDER, S. H. 1995. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *The Journal of Cell Biology,* 130**,** 857-869.

WALSCHAERTS, M., BUJAN, L., PARINAUD, J., MIEUSSET, R. & THONNEAU, P. 2013. Treatment discontinuation in couples consulting for male infertility after failing to conceive. *Fertility and Sterility*.

WANG, G., GUO, Y., ZHOU, T., SHI, X., YU, J., YANG, Y., WU, Y., WANG, J., LIU, M., CHEN, X., TU, W., ZENG, Y., JIANG, M., LI, S., ZHANG, P., ZHOU, Q., ZHENG, B., YU, C., ZHOU, Z., GUO, X. & SHA, J. 2013. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *Journal of proteomics,* 79**,** 114-22.

WANG, H., DEY, S. K. & MACCARRONE, M. 2006. Jekyll and Hyde: Two Faces of Cannabinoid Signaling in Male and Female Fertility. *Endocrine Reviews,* 27**,** 427-448.

WEIDINGER, S., MAYERHOFER, A., KUNZ, L., ALBRECHT, M., SBORNIK, M., WUNN, E., HOLLWECK, R., RING, J. & KOHN, F. M. 2005. Tryptase inhibits motility of human spermatozoa mainly by activation of the mitogen-activated protein kinase pathway. *Human Reproduction,* 20**,** 456-461.

WELSBY, P. J., WANG, H., WOLFE, J. T., COLBRAN, R. J., JOHNSON, M. L. & BARRETT, P. Q. 2003. A Mechanism for the Direct Regulation of T-Type Calcium Channels by Ca2+/Calmodulin-Dependent Kinase II. *The Journal of Neuroscience,* 23**,** 10116-10121.

WENNEMUTH, G., CARLSON, A. E., HARPER, A. J. & BABCOCK, D. F. 2003. Bicarbonate actions on flagellar and Ca2+-channel responses: initial events in sperm activation. *Development,* 130**,** 1317-1326.

WHO, W. H. O. 1987. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge University Press: New York, New York, USA; Cambridge, England, Uk. Illus. Paper*.

WHO, W. H. O. 1992. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction,* Cambridge, Cambridge University Press.

WHO, W. H. O. 2010a. Induced acrosome reaction assay. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction / Edition 5.* 5 ed.: World Health Organization.

WHO, W. H. O. 2010b. Reference values and semen nomenclature.

WHO, W. H. O. 2010c. Semen Analysis: Preparing the Samples. *WHO Laboratory Manual for Examintation of Human Semen and Sperm-Cervical Mucus Interaction/ Edition 5.* 5 ed.: World Health Organization.

WHO, W. H. O. 2010d. WHO Laboratory Manual for Examination of Human Semen and Sperm-Cervical Mucus Interaction/ Edition 5. 5 ed.: World Health Organization.

WHO, W. H. O. 2010e. *WHO laboratory manual for the Examination and processing of human semen,* Switzerland, World Health Organization.

WHO, W. H. O. 2010f. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction / Edition 5. 5 ed.: World Health Organization.

WILLIAMS, A. C. & FORD, W. C. L. 2001. The Role of Glucose in Supporting Motility and Capacitation in Human Spermatozoa. *Journal of Andrology,* 22**,** 680-695.

WILTSHIRE, E. J., FLAHERTY, S. P. & COUPER, R. T. L. 2000. Hepatocyte growth factor in human semen and its association with semen parameters. *Human Reproduction,* 15**,** 1525-1528.

WONG, C.-H. & YAN CHENG, C. 2005. Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: A review of recent data. *Developmental Biology,* 286**,** 1-15.

XIA, J., REIGADA, D., MITCHELL, C. H. & REN, D. 2007. CATSPER Channel-Mediated Ca2+ Entry into Mouse Sperm Triggers a Tail-to-Head Propagation. *Biology of Reproduction,* 77**,** 551-559.

XIE, F., EDDY, E. M. & CONTI, M. 2013. Chapter Six - Analysis of Signaling Pathways Controlling Flagellar Movements in Mammalian Spermatozoa. *In:* WALLACE, F. M. (ed.) *Methods in Enzymology*. Academic Press.

XIE, F., GARCIA, M. A., CARLSON, A. E., SCHUH, S. M., BABCOCK, D. F., JAISWAL, B. S., GOSSEN, J. A., ESPOSITO, G., VAN DUIN, M. & CONTI, M. 2006. Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. *Developmental Biology,* 296**,** 353-362.

YAN, C., ZHAO, A. Z., SONNENBURG, W. K. & BEAVO, J. A. 2001. Stage and Cell-Specific Expression of Calmodulin-Dependent Phosphodiesterases in Mouse Testis. *Biology of Reproduction,* 64**,** 1746-1754.

YANAGIMACHI, R. 1970. The movement of golden hamster spermatozoa before and after capacitation. *Journal of Reproduction and Fertility,* 23**,** 193-196.

YANAGIMACHI, R. 1994. *Mammalian fertilisation,* New York, Raven Press.

YAO, Y. Q., NG, V., YEUNG, W. S. B. & HO, P. C. 1996. Profiles of sperm morphology and motility after discontinuous multiple-step Percoll density gradient centrifugation. *Andrologia,* 28**,** 127-131.

YOVICH, J., EDIRISINGHE, W., CUMMINS, J. & YOVICH, J. 1990. Influence of pentoxifylline in severe male factor infertility. *Fertility and Sterility,* 53**,** 715.

YOVICH, J. L. 1993. PENTOXIFYLLINE - ACTIONS AND APPLICATIONS IN ASSISTED REPRODUCTION. *Human Reproduction,* 8**,** 1786-1791.

YUASA, K., KANOH, Y., OKUMURA, K. & OMORI, K. 2001. Genomic organization of the human phosphodiesterase PDE11A gene. *European Journal of Biochemistry,* 268**,** 168-178.

ZHOU, C.-X., WANG, X.-F. & CHAN, H.-C. 2005. Bicarbonate secretion by the female reproductive tract and its impact on sperm fertilizing capacity. *Shengli Xuebao,* 57**,** 115-124.

ZHU, M. X., MA, J., PARRINGTON, J., CALCRAFT, P. J., GALIONE, A. & EVANS, A. M. 2010a. Calcium signaling via two-pore channels: local or global, that is the question. *American Journal of Physiology - Cell Physiology,* 298**,** C430-C441.

ZHU, M. X., MA, J., PARRINGTON, J., GALIONE, A. & MARK EVANS, A. 2010b. TPCs: Endolysosomal channels for Ca2+ mobilization from acidic organelles triggered by NAADP. *FEBS Letters,* 584**,** 1966-1974.

CHAPTER 10

Appendix

10.1 Components, product list and catalogue numbers for Media and Compounds used.

Compound	NCM (mM)	CM (mM)	Source	Catalogue N^{o}
$CaCl_2$	1.8	1.8	Sigma-Aldrich	C3306 - 100
KCl	5.4	5.4	Sigma-Aldrich	P5405 – 250g
$MgSO_4.7H_2O$	0.8	0.8	Sigma-Aldrich	M2773 - 500g
Sodium chloride	116.4	116.4	Sigma-Aldrich	S5886 – 1Kg
$NaH_2PO_4.2H_2O$	1.0	1.0	Sigma-Aldrich	71505 - 250g
D- glucose	5.55	5.55	Sigma-Aldrich	G6152 – 500g
Sodium lactate	41.75	25	Sigma-Aldrich	L7900 – 100ml
Sodium pyruvate	2.73	2.73	Sigma-Aldrich	P2256 – 25g
HEPES	25	-	Sigma-Aldrich	H3375 – 1Kg
Sodium bicarbonate	-	26	Sigma-Aldrich	S5761 -500g
BSA	0.3%	0.3%	Sigma-Aldrich	A3059 – 100g

Compound	Source	Catalogue N^{o}
DMSO	Sigma-Aldrich	673439
PHA665752	Tocris	2693
Leelamine Hydrochloride	Tocris	2139
GP1a	Tocris	2764
JX401	Tocris	2657
EO1428	Tocris	2908
Trequinsin Hydrochloride	Tocris	2337
PureSperm 40% and 80%	Hunter Scientific	PS40-100 PS80-100
SAGE Gamete buffer	Origio	ART-1005
Quinns Advantage Fertilisation Medium	Origio	ART-1020
Human Serum Albumin (HSA)	Origio	ART-3001
Percoll	Sigma-Aldrich	P1644
Calcium 3	Molecular Devices	
Methylcellulose	Sigma-Aldrich	M0512
PSA FITC	Sigma-Aldrich	L0770
Calcium Ionophore	Sigma-Aldrich	C7522
PBS	Sigma-Aldrich	P4417
Hydromount	National Diagnostics	HS-106
D-fructose	Sigma-Aldrich	F0127
Sodium citrate dihydrate	Sigma-Aldrich	W302600

Table 10.1 Identification of compounds and the product source for media which were utilised in all experiments conducted within this project.

10.2 Comparing donor fractions to 80% fraction recovered from patients.

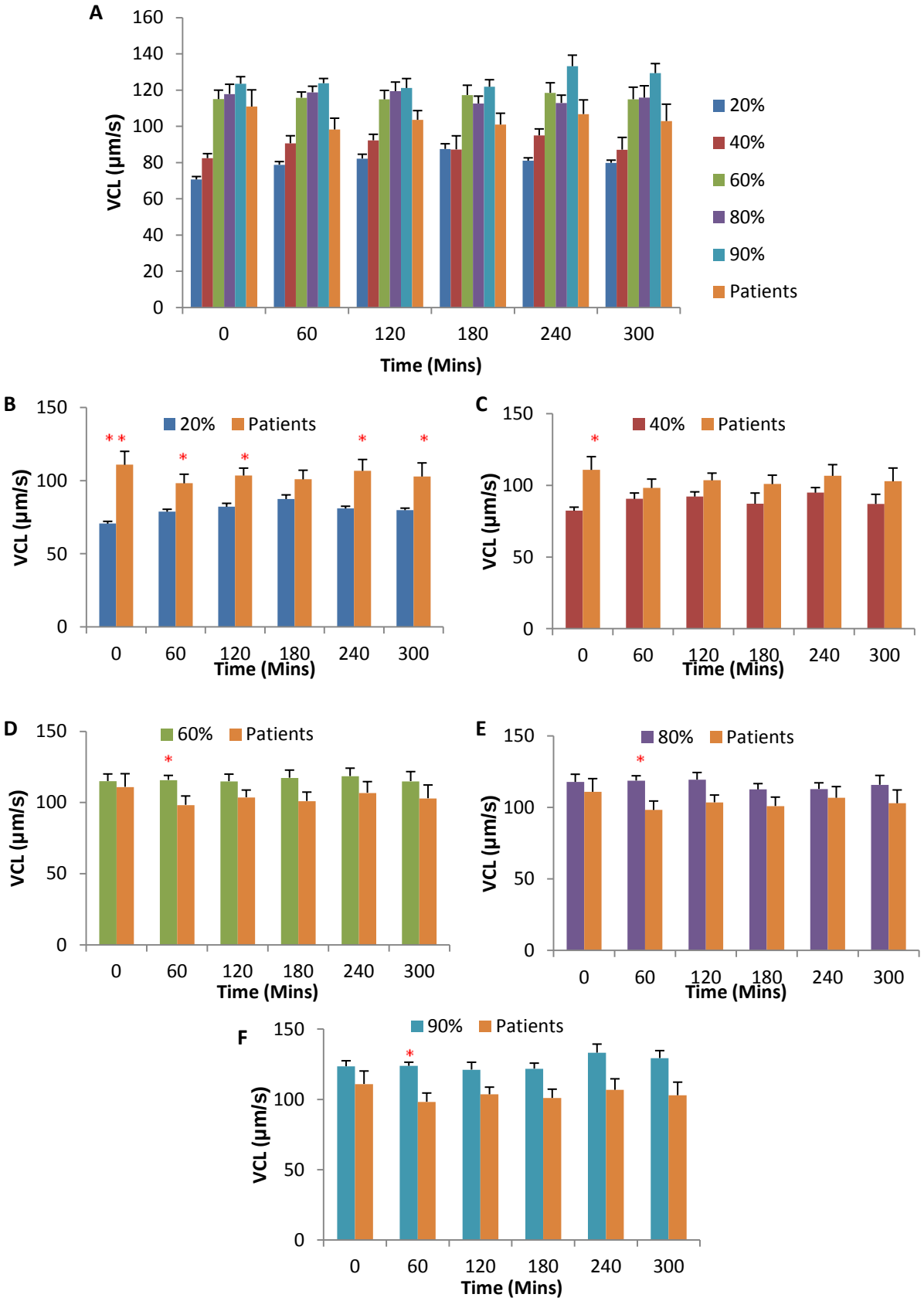


Figure 10-2. Comparison between donor and sub-fertile samples in Curvelinear Velocity (VCL) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05)* **indicates *P<0.01* (T-test and Kruskall-Wallace test).

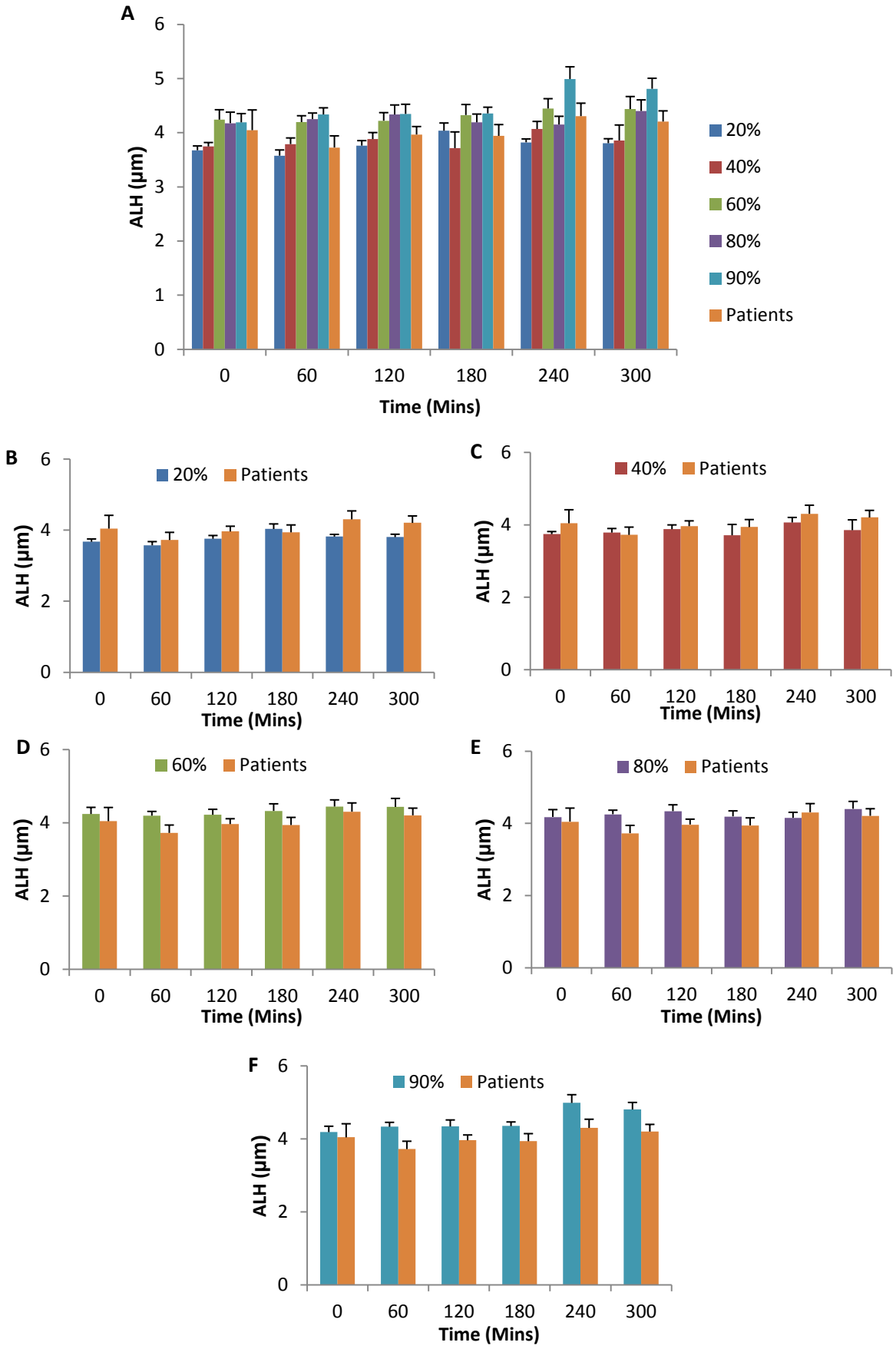


Figure 10-2. Comparison between donor and sub-fertile samples in amplitude of lateral head displacement (ALH) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05)* (Mann Whitney test and Kruskall-Wallace test).

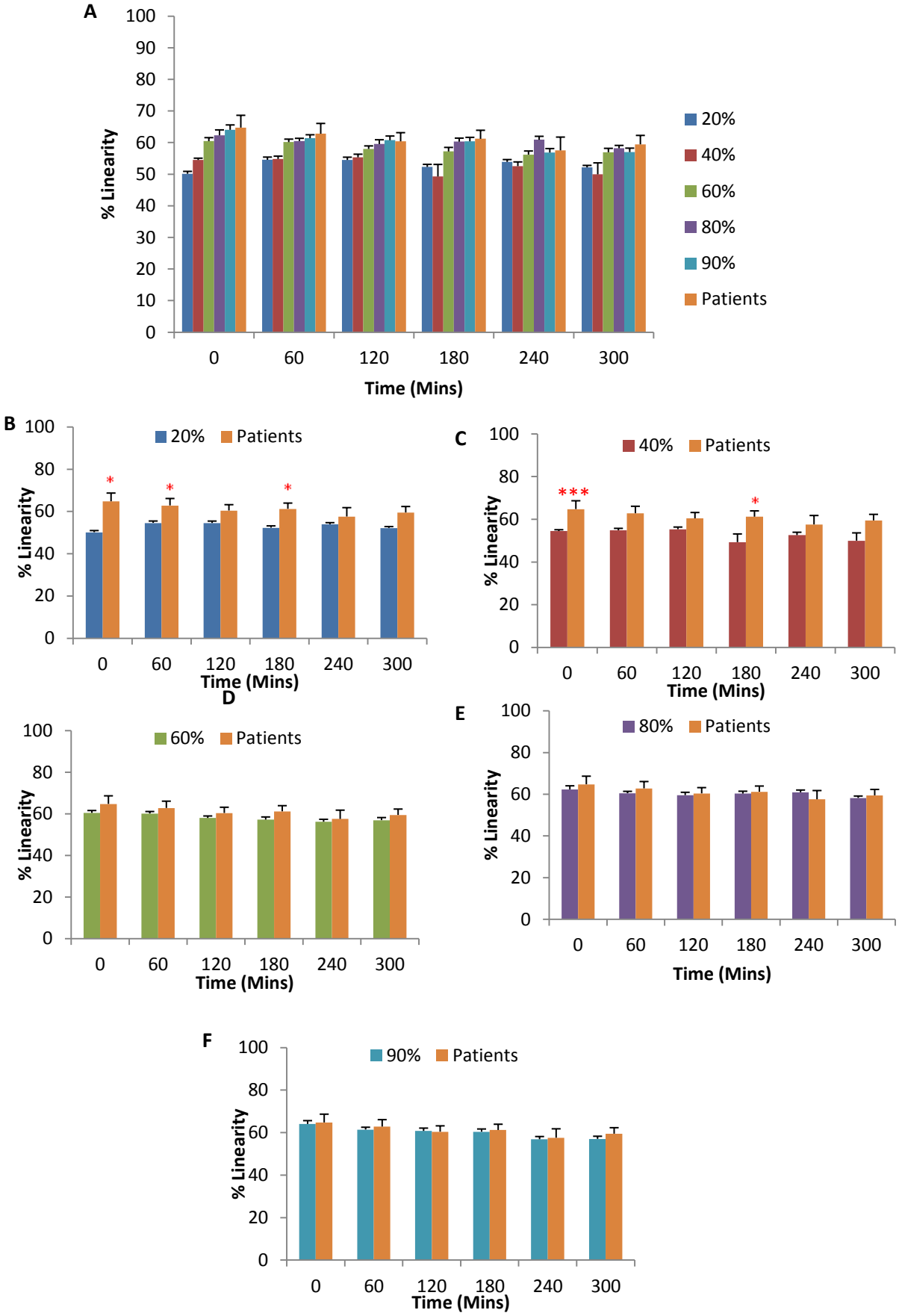


Figure 10-3. Comparison between donor and sub-fertile samples in linearity (LIN) in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05*) ***indicates *P<0.001* (T-test and Kruskall-Wallace test).

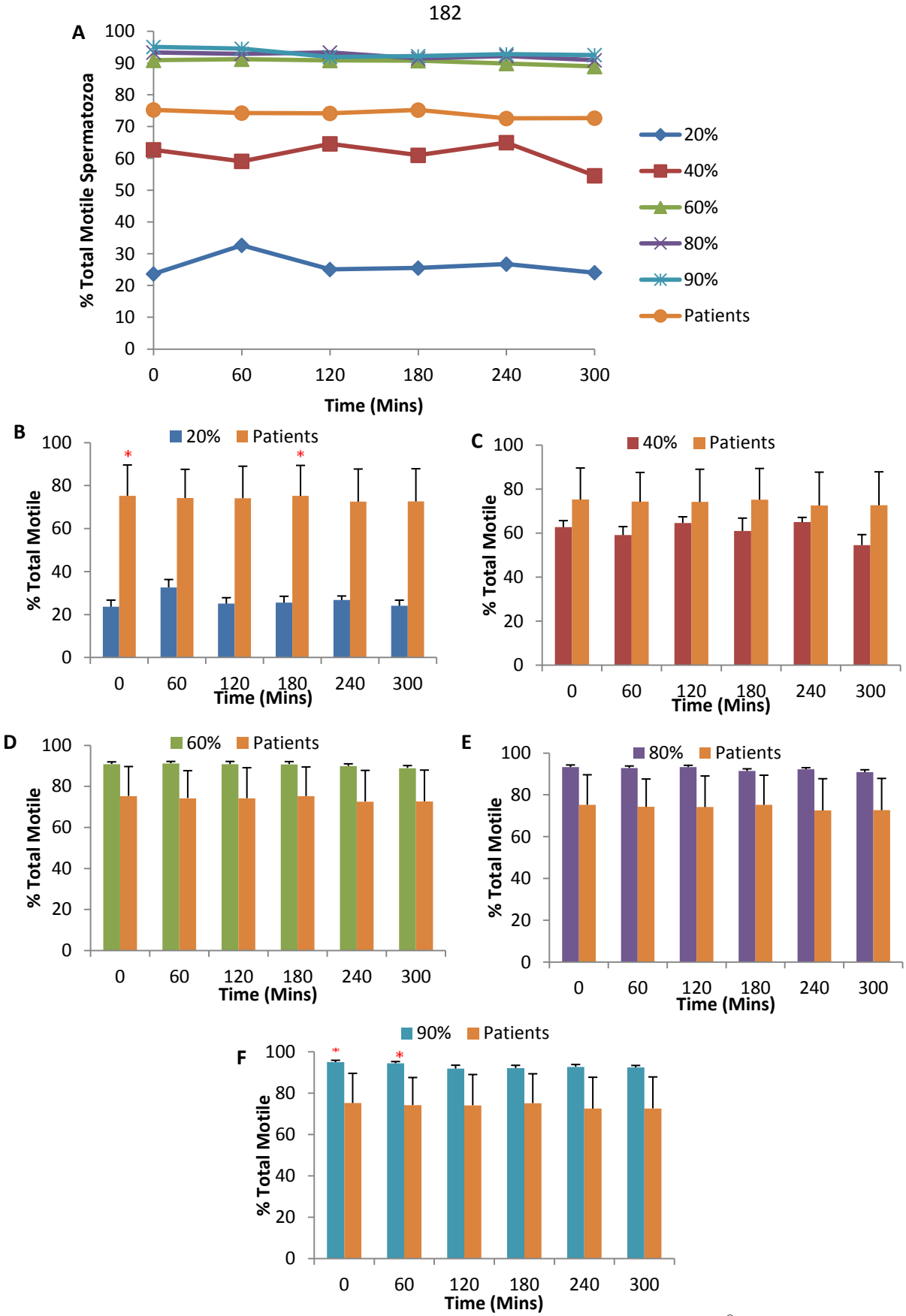


Figure 10-4. Comparison between donor and sub-fertile samples in % total motile in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05)* (Mann Whitney test and Kruskall-Wallace test).

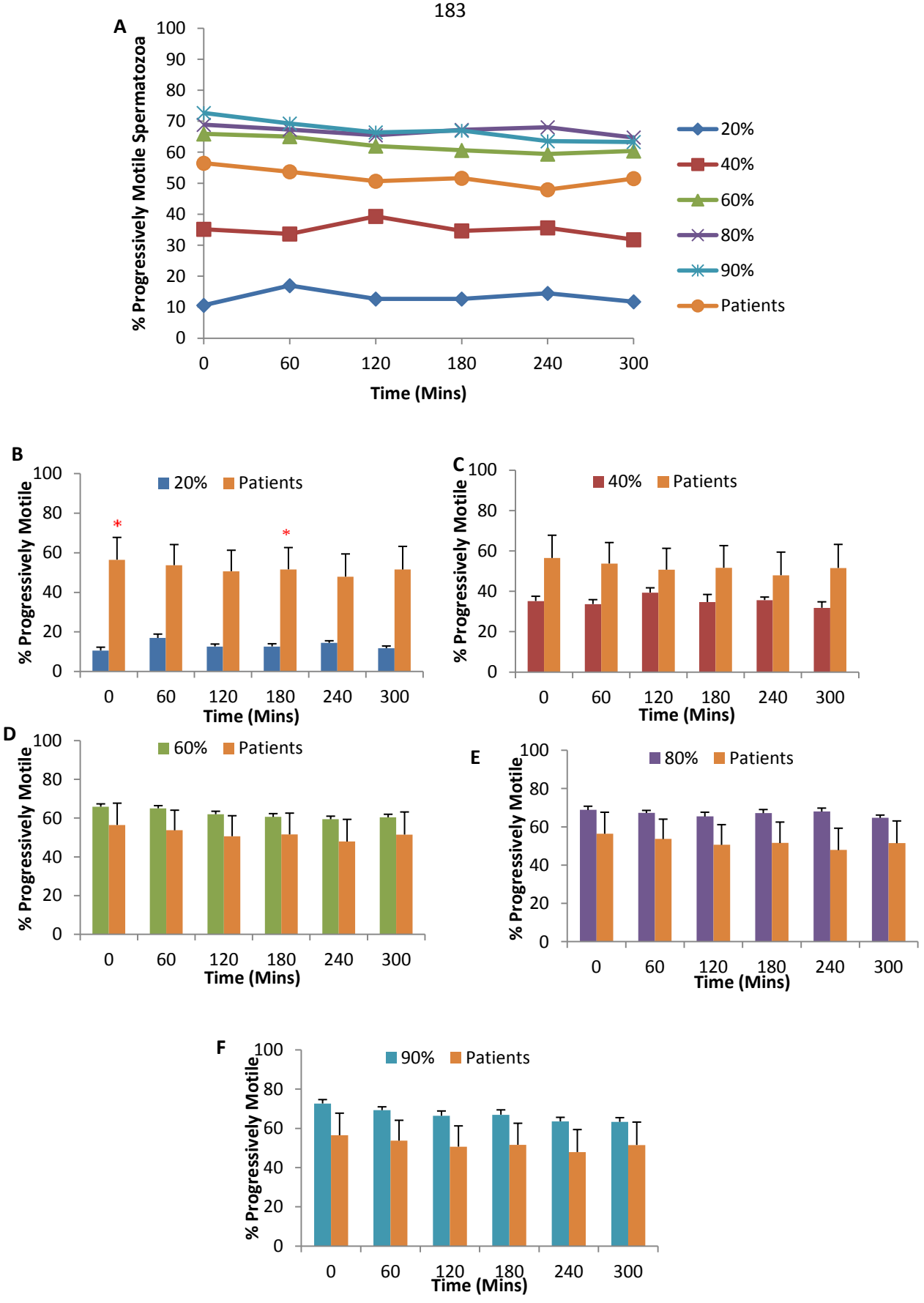


Figure 10-5. Comparison between donor and sub-fertile samples in % progressive motility in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05)* (Mann Whitney test and Kruskall-Wallace test).

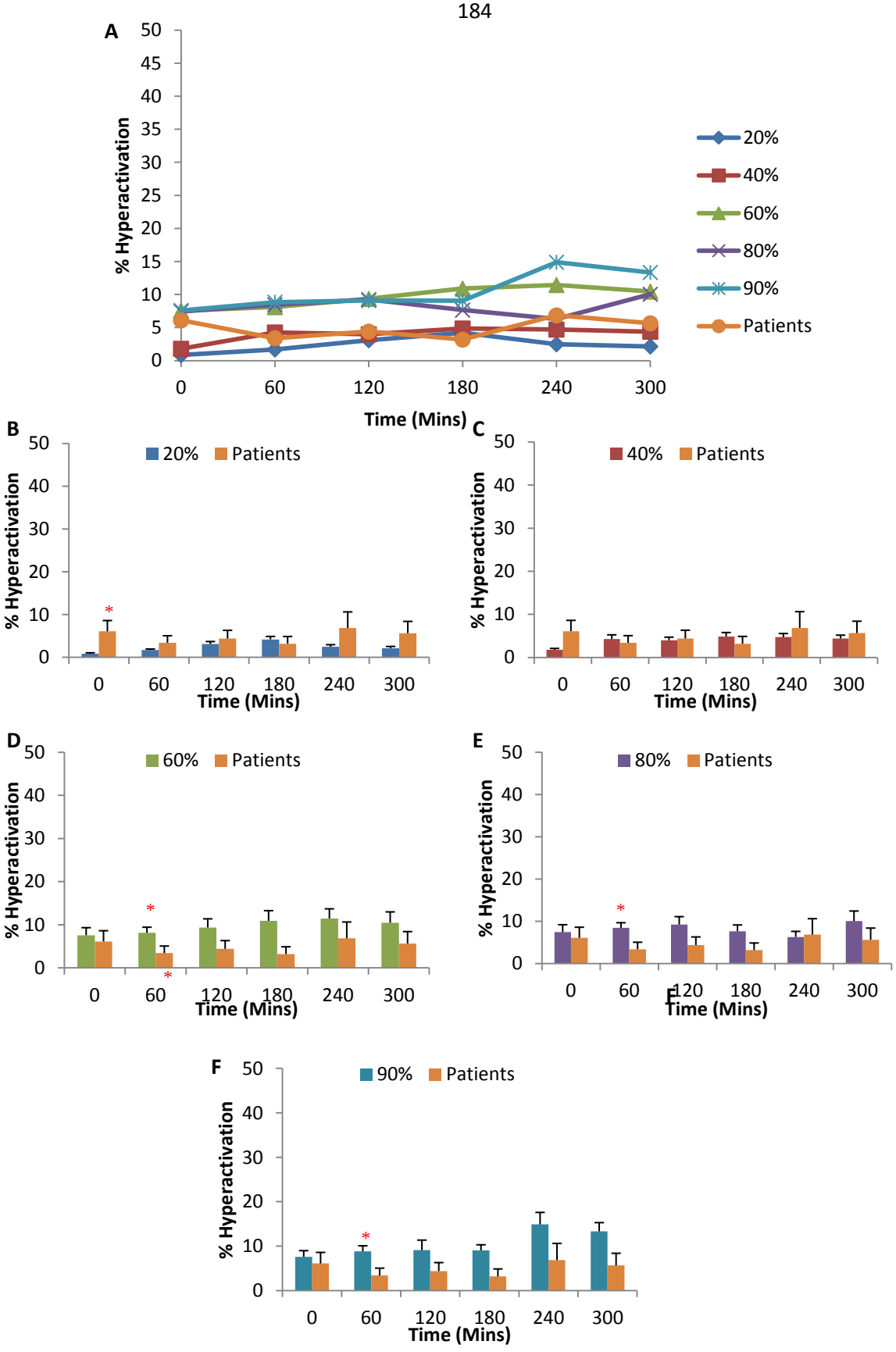


Figure 10-6. Comparison between donor and sub-fertile samples in % hyperactivation in Percoll® fractions relative to incubation time under capacitating conditions (A) Overview of 20%, 40%, 60%, 80% and 90% donor fraction compared to 80% sub-fertile fraction **(B)** 20% donor, 80% sub-fertile **(C)** 40% donor, 80% sub-fertile **(D)** 60% donor, 80% sub-fertile **(E)** 80% donor, 80% sub-fertile **(F)** 90% donor, 80% sub-fertile. The result shown is the mean ± SEM. N= 5 for both donors and patients, *indicates significant difference (*P<0.05)* (Mann Whitney test and Kruskall-Wallace test).

10.3 Determination of the number of aliquots from a single sample that need to be taken to assess motility parameters

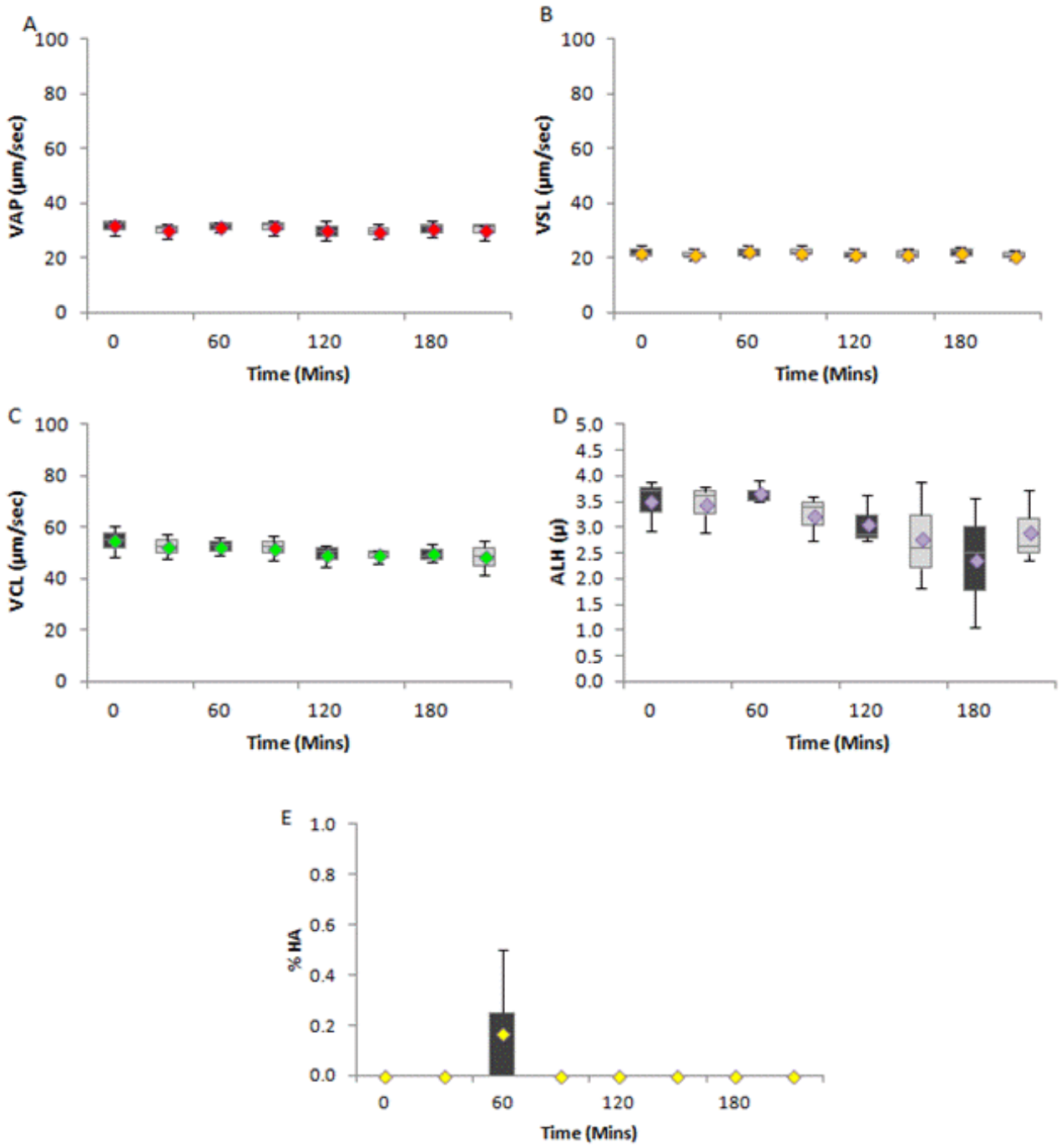


Figure 10-7 Variation in range of results, 4 aliquots vs 2 aliquots, for the 40% fraction of spermatozoa in non-capacitating buffer (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH and **(E)** % hyperactivation. The result shown is the mean (coloured diamonds) ± the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

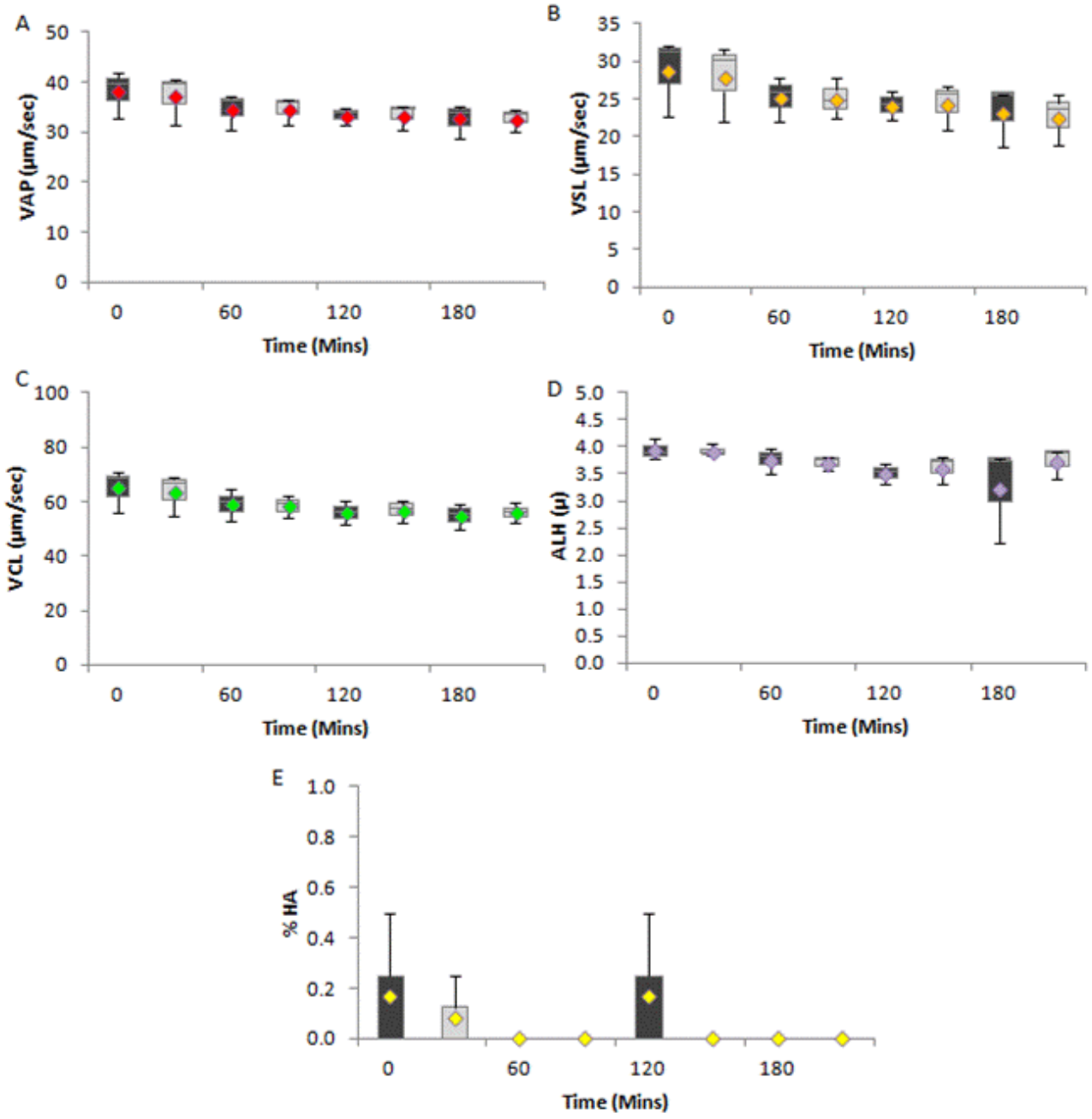


Figure 10-8 Variation in range of results, 4 aliquots vs 2 aliquots, for the 80% fraction of spermatozoa in non-capacitating buffer (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH and **(E)** % hyperactivation. The result shown is the mean (coloured diamonds) ± the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

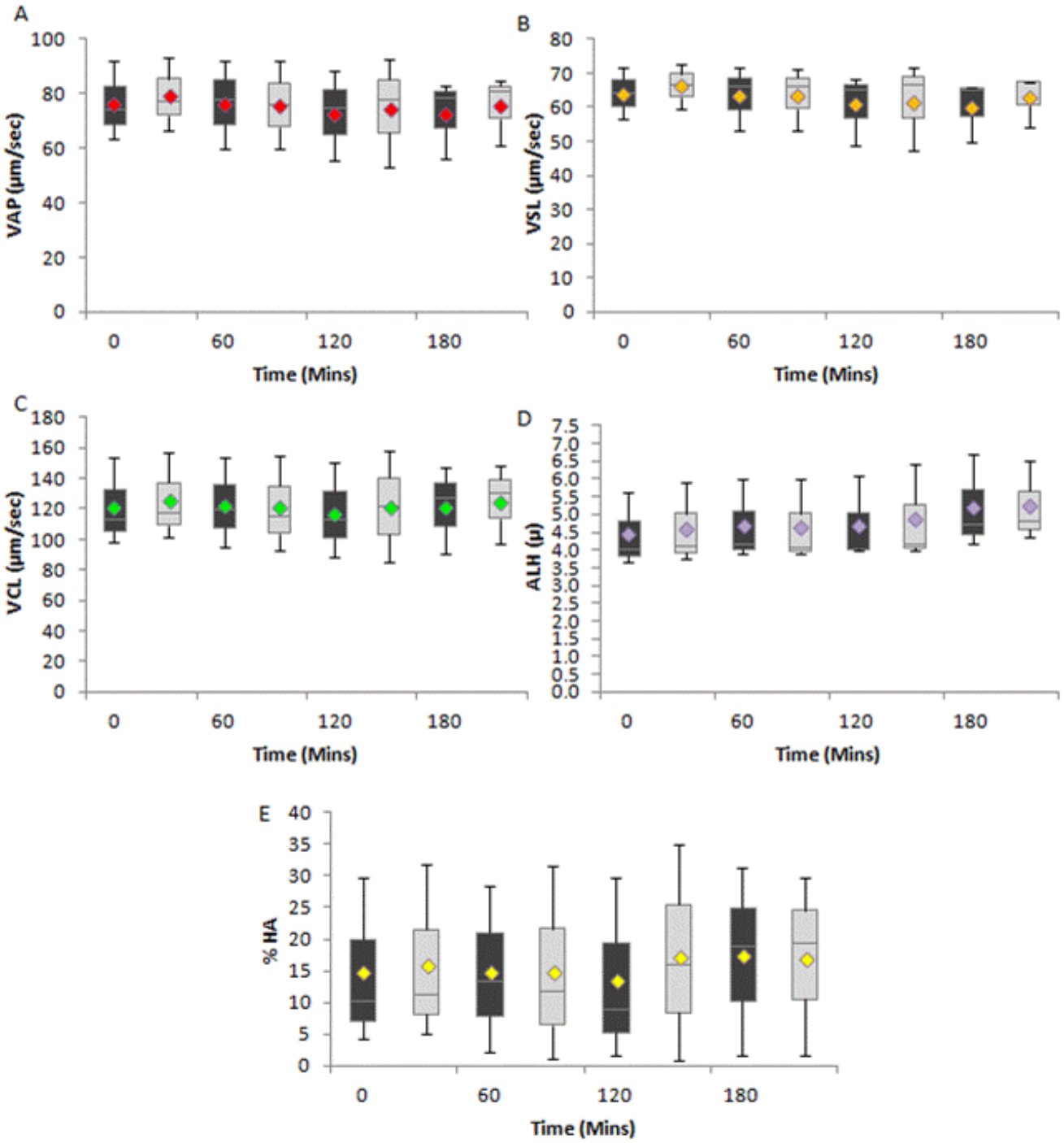


Figure 10-9 Variation in range of results, 4 aliquots vs 2 aliquots, for the 40% fraction of spermatozoa in capacitating conditions (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH and **(E)** % hyperactivation. The result shown is the mean (coloured diamonds) ± the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

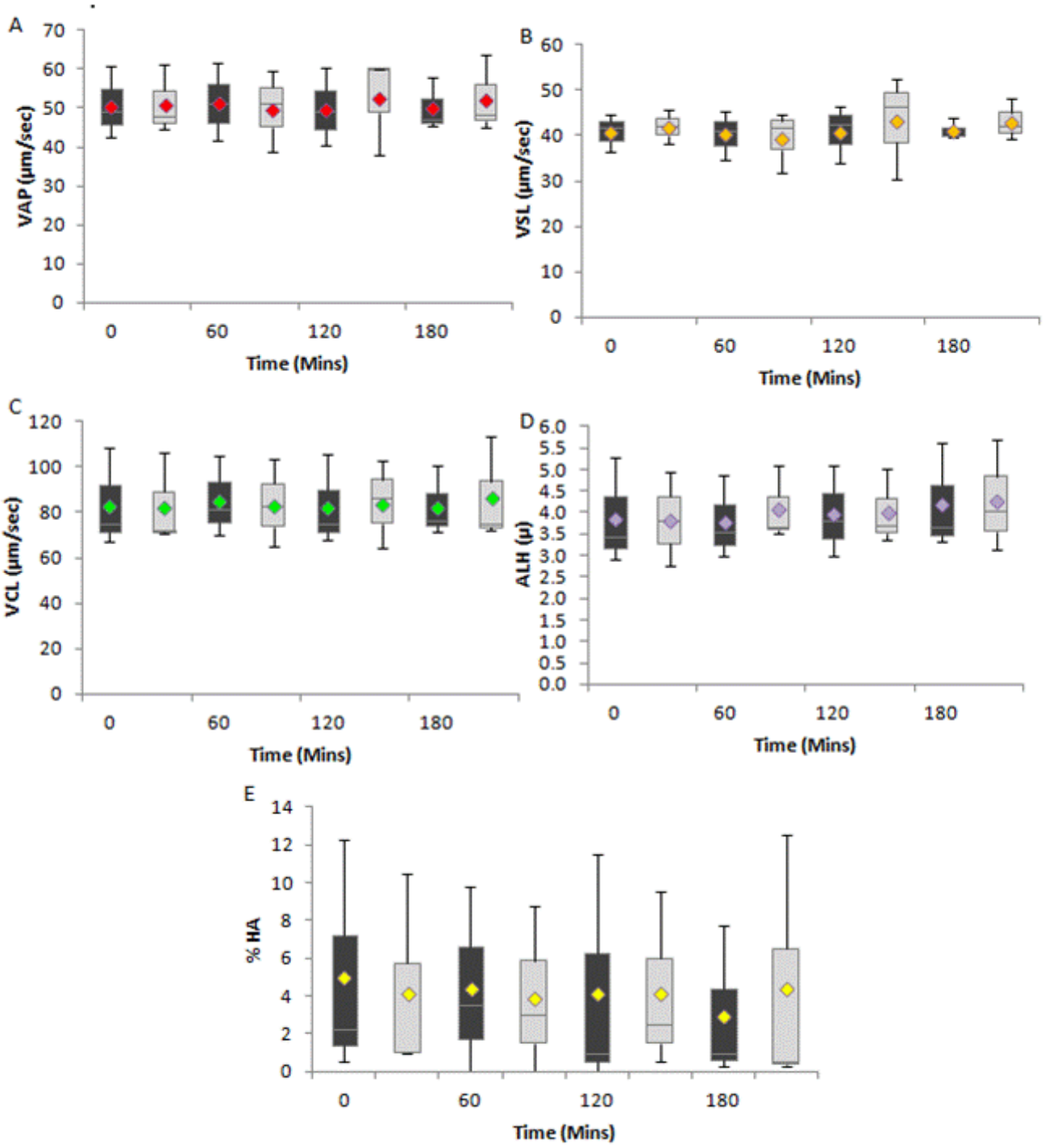


Figure 10-10 Variation in range of results, 4 aliquots vs 2 aliquots, for the 80% fraction of spermatozoa in capacitating conditions (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH and **(E)** % hyperactivation. The result shown is the mean (coloured diamonds) ± the range for A-E, see figure 4-1 for identifying features of box plots. Motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the number of replicates needed to produce a low sampling error when measuring sperm motility. Dark Grey boxes = 2 aliquots, 400 cells per aliquot, Light Grey boxes = 4 aliquots, 200 cells per aliquot. N= 3 for both parameters. Results show no significant differences (Mann Whitney test)

10.4 Determination of slide type required for CASA assessment of motility

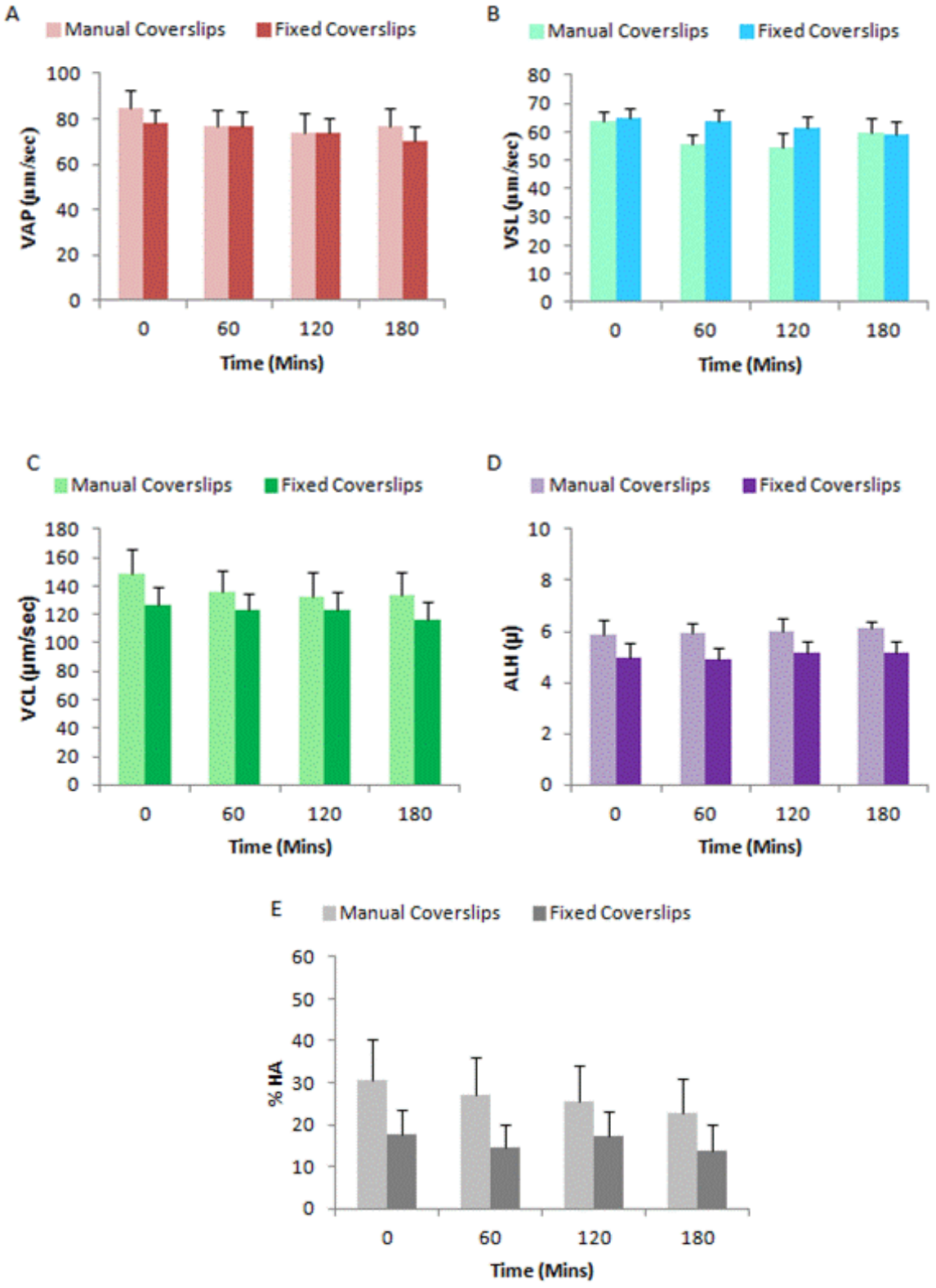


Figure 10-11 Variation in motility between two slide types: Hamilton Thorne glass slides with manual glass coverslips and fixed chamber slides (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** % hyperactivation. The result shown is the mean ± SE measured at defined time points up to 180 min N= 3 for both parameters, * indicates a significant increase between manual and fixed slides at independent time points for 80% fraction of sperm under capacitating conditions. Significance was considered as *P<0.05*

10.5 Assessment of motility parameters in capacitating and non-capacitating media

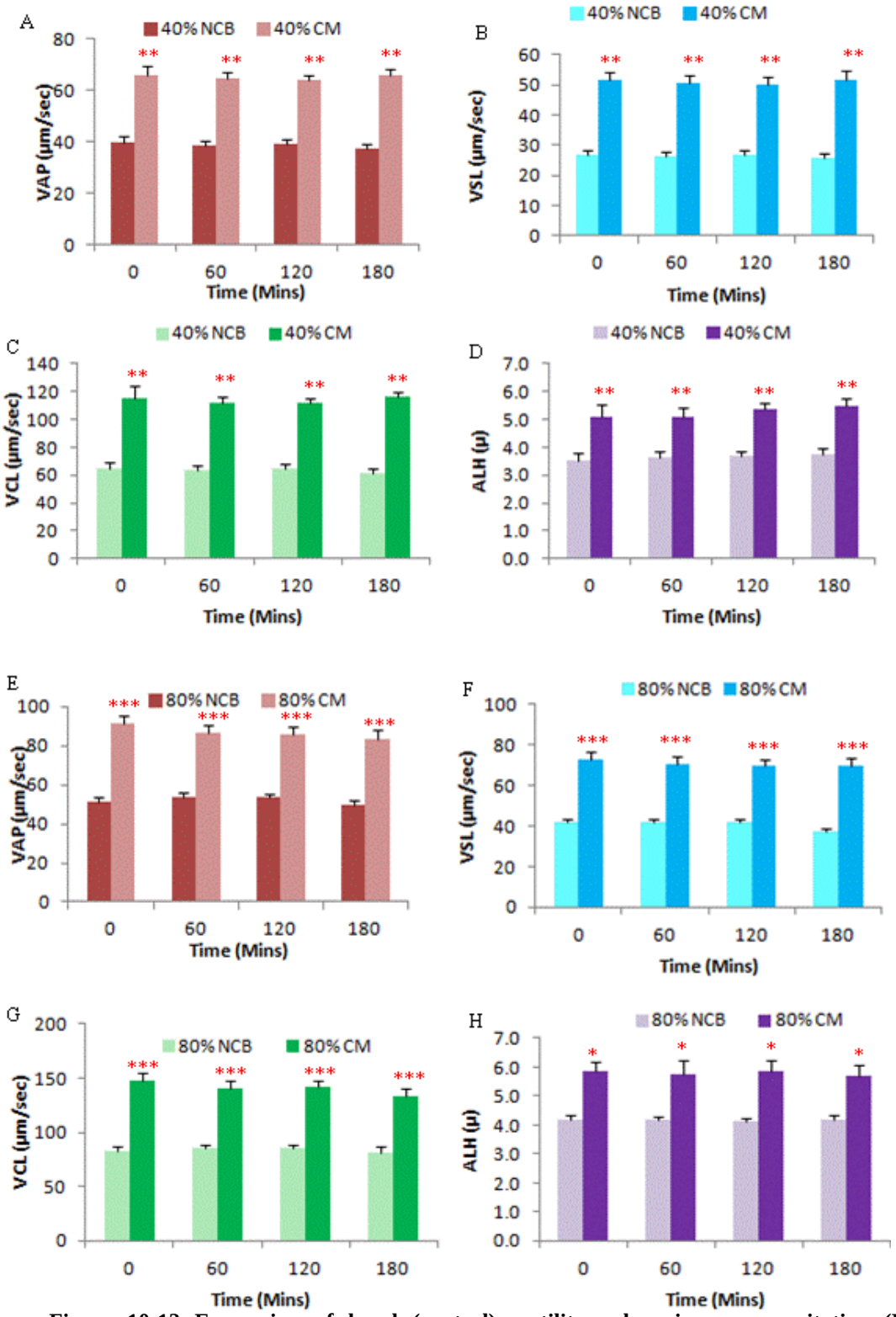


Figure 10-12 Expression of basal (control) motility values in non-capacitating (NCM) and capacitating media (CM) for 40%; (A) VAP **(B)** VSL **(C)** VCL and **(D)** ALH **and 80% fractions; (E)** VAP, **(F)** VSL, **(G)** VCL and **(H)** ALH. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating and capacitating conditions comparing the difference between the two media types. N= 5 for NCM and N= 9 for CM, *** indicates a significant increase (*P<0.005* or *P<0.001)* between NCM and CM at independent time points (Mann Whitney test and Kruskal-Wallace test).

10.6 Effect of 1% DMSO on treated sperm samples

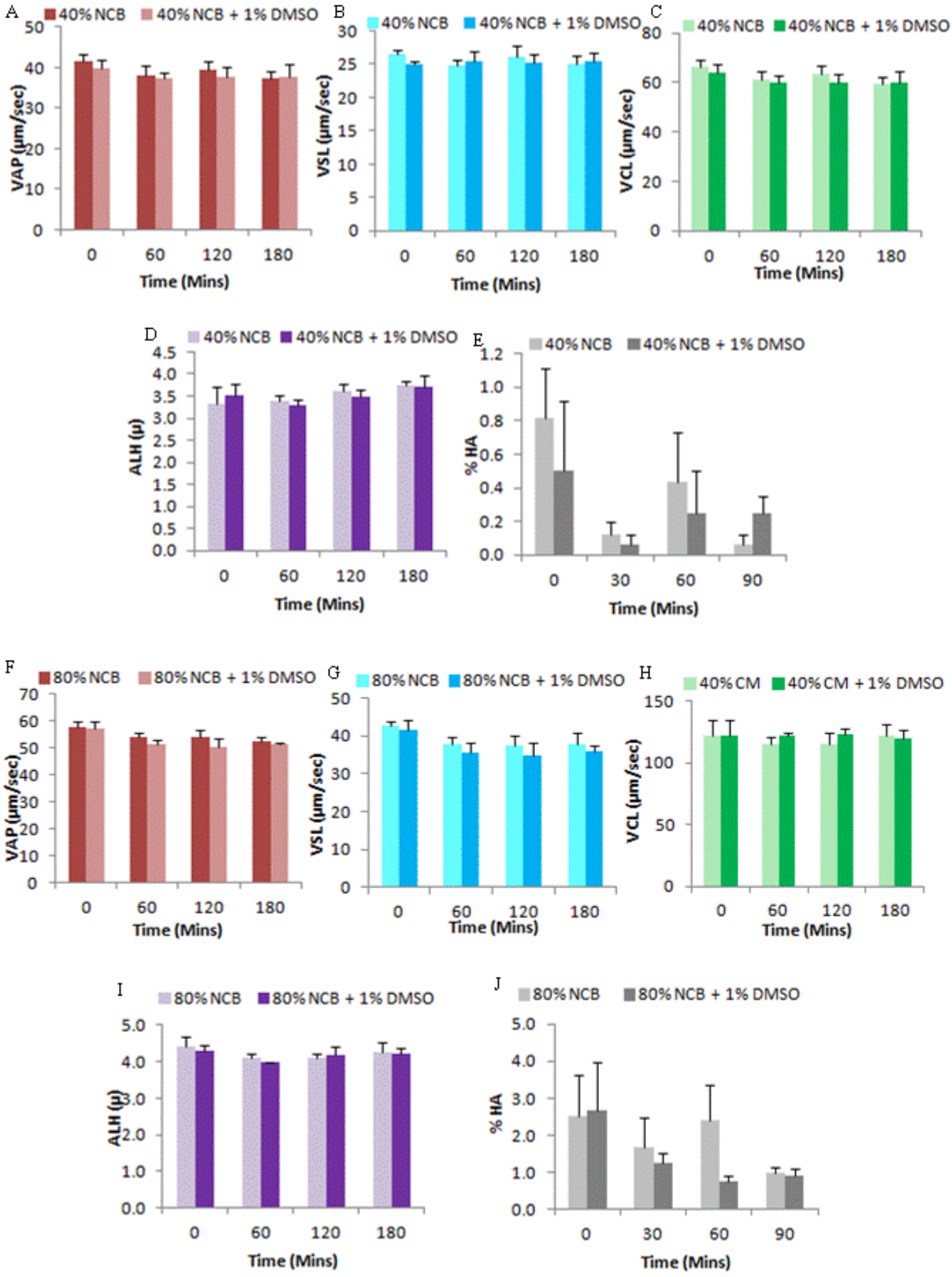


Figure 10-13 Expression of basal (control) motility values in non-capacitating media (NCM) treated with 1% DMSO in 40% fraction (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH, **(E)** % HA and **80% fraction (F)** VAP, **(G)** VSL, **(H)** VCL, **(I)** ALH, **(J)** % HA. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non- capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 4 for NCM and NCM + 1% DMSO, no significant difference noted at any time point.

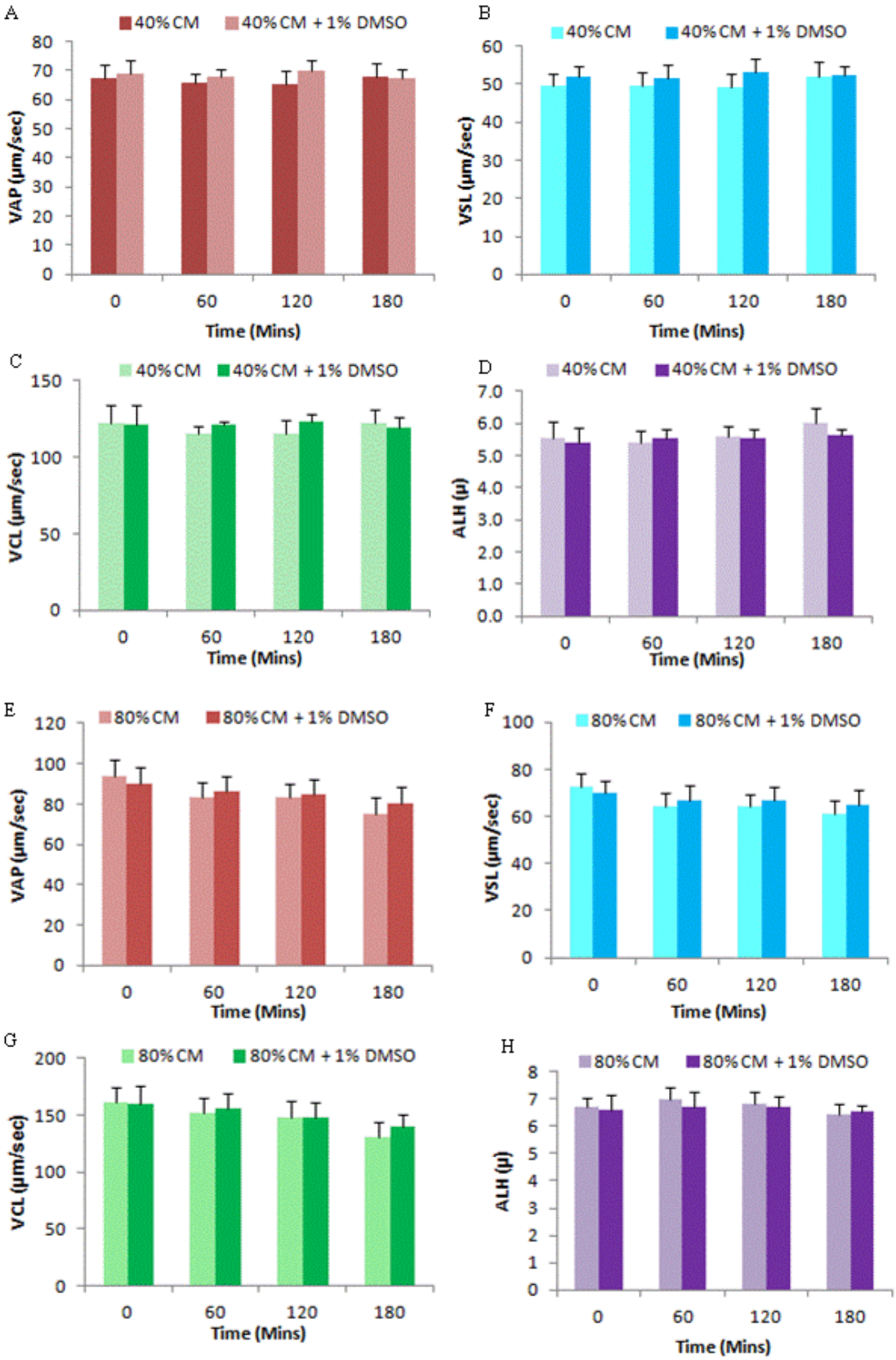


Figure 10-14 Expression of basal (control) motility values capacitating media (CM) treated with 1% DMSO in 40% fraction (A) VAP, **(B)** VSL, **(C)** VCL, **(D)** ALH and **80% fraction (E)** VAP, **(F)** VSL, **(G)** VCL, **(H)** ALH. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between treatment with 1% DMSO and non-treated media. N= 4 for CM and CM + 1% DMSO, no significant difference noted at any time point.

10.7 Treatment with Leelamine Hydrochloride

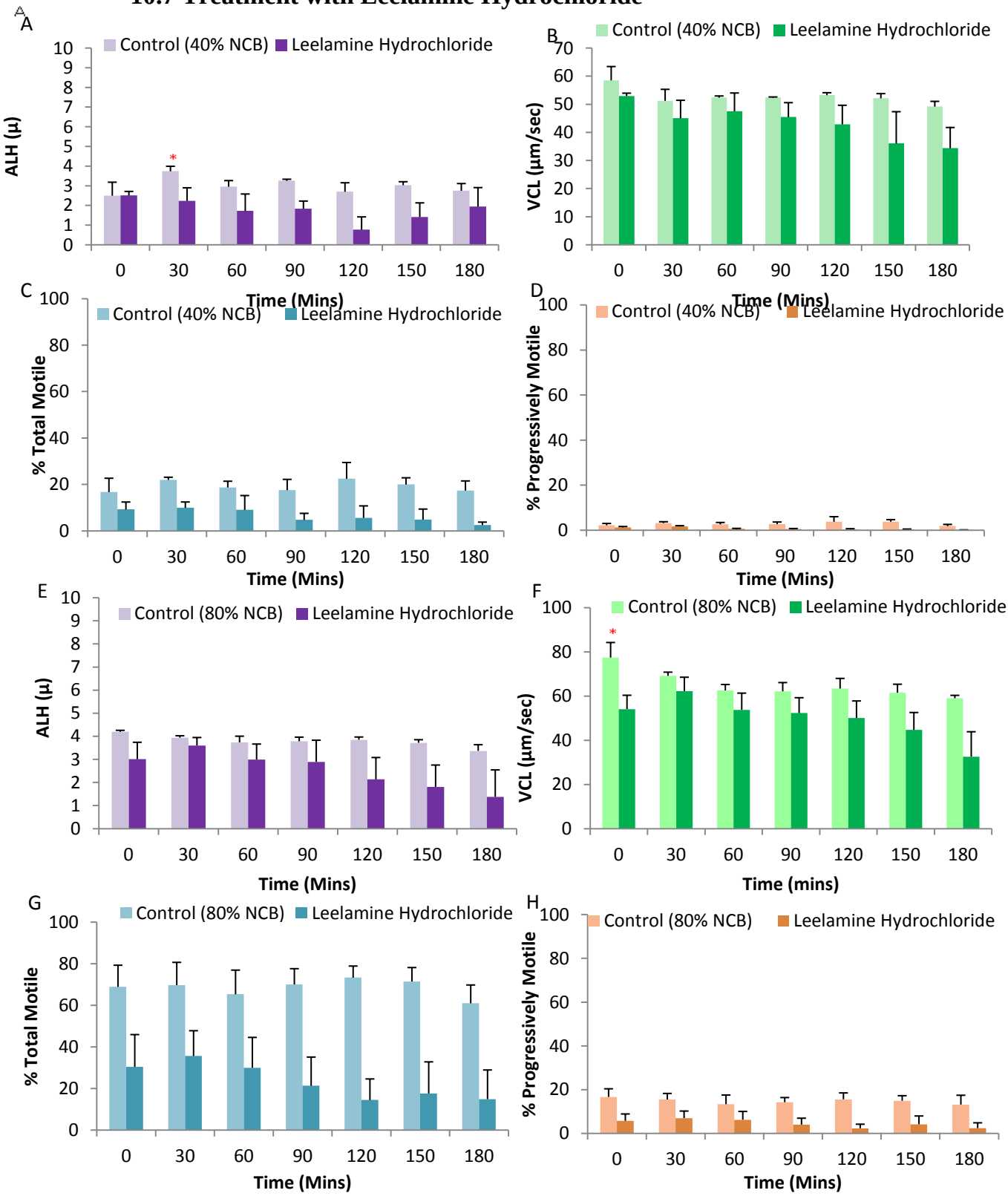


Figure 10-15 Expression of basal (control) motility values compared to treated samples (Leelamine Hydrochloride) in non-capacitating (NCM) media, 40% fraction (A) ALH, **(B)** VCL, **(C)** % total motile, **(D)** % progressively motile **and 80% fraction (E)** ALH, **(F)** VCL, **(G)** % total motile, **(H)** % progressively motile. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

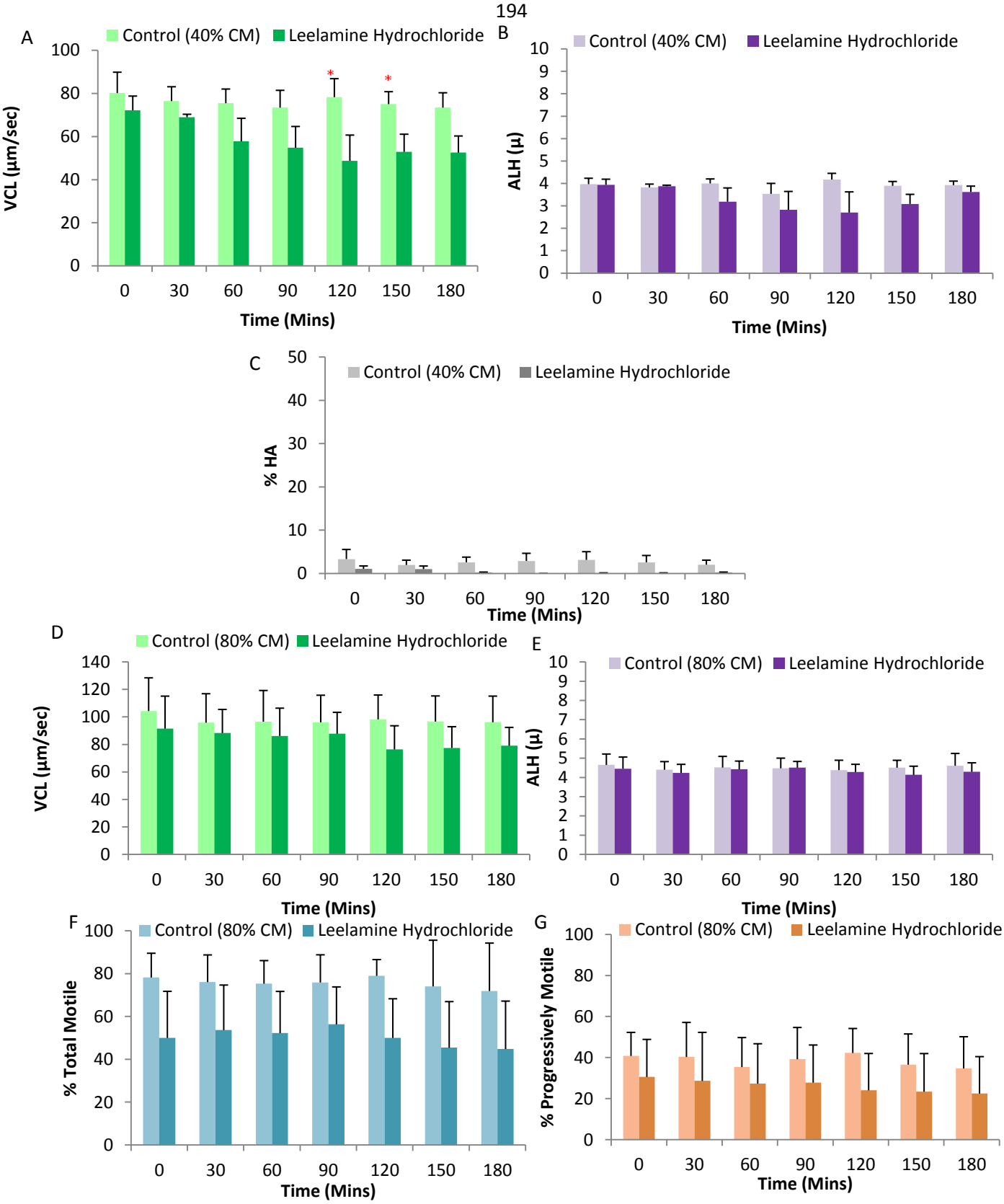


Figure 10-16 Expression of basal (control) motility values compared to treated samples (Leelamine Hydrochloride) in capacitating (CM) media, 40% fraction (A) VCL, **(B)** ALH, **(C)** % HA **and 80% fraction (D)** VCL, **(E)** ALH, **(F)** % total motile, **(G)** % progressively motile. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 4 for both parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

10.8 Treatment with GP1a

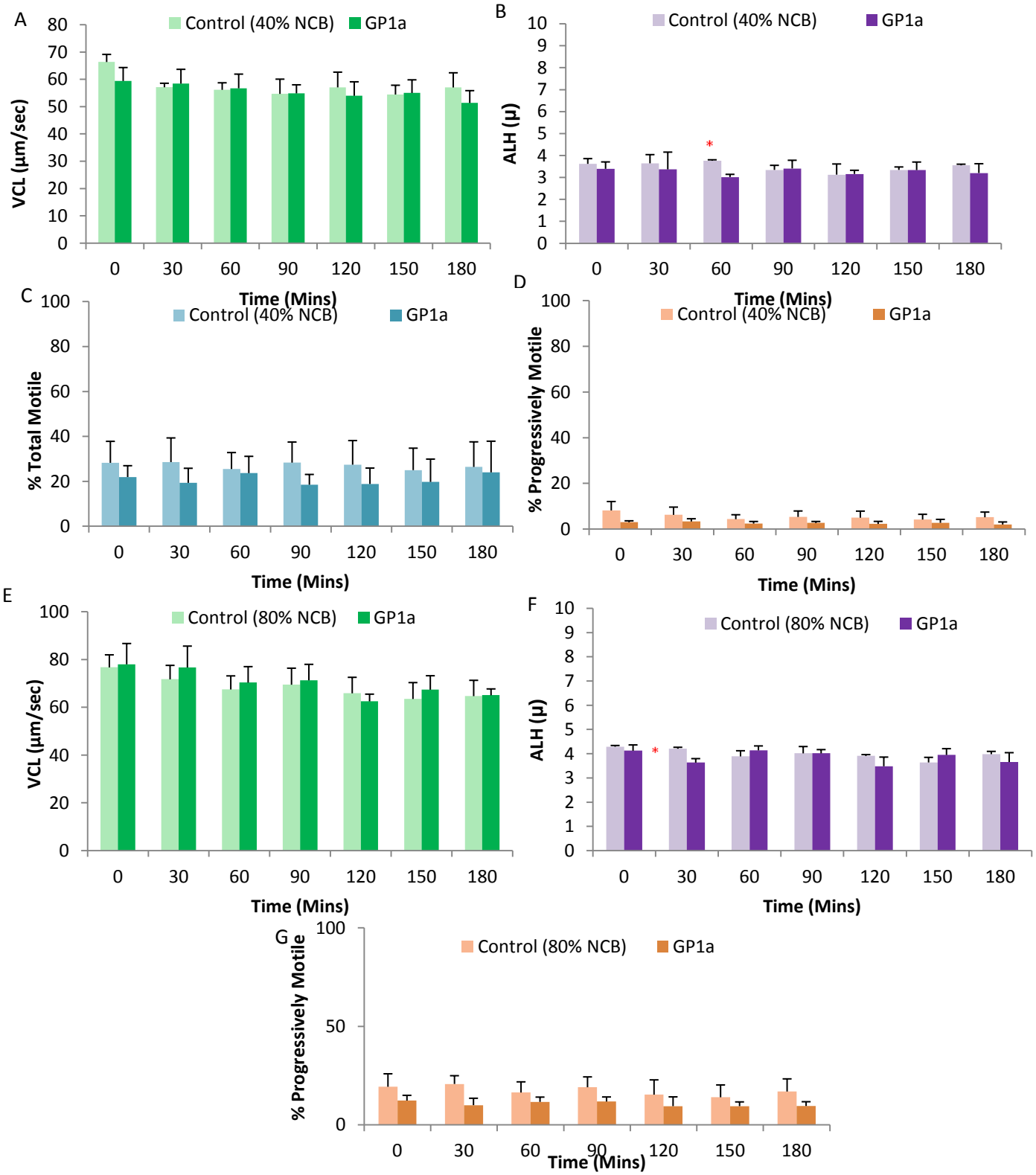


Figure 10-17 Expression of basal (control) motility values compared to treated samples (GP1a) in non- capacitating (NCM) media (40% and 80% fraction). For 40% fraction (A) VCL**, (B)** ALH, **(C)** % total motile, **(D)** % progressively motile **and for 80% fraction (E)** VCL, **(F)** ALH and **(G)** % progressive motility. The result shown is the mean ± SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non- capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

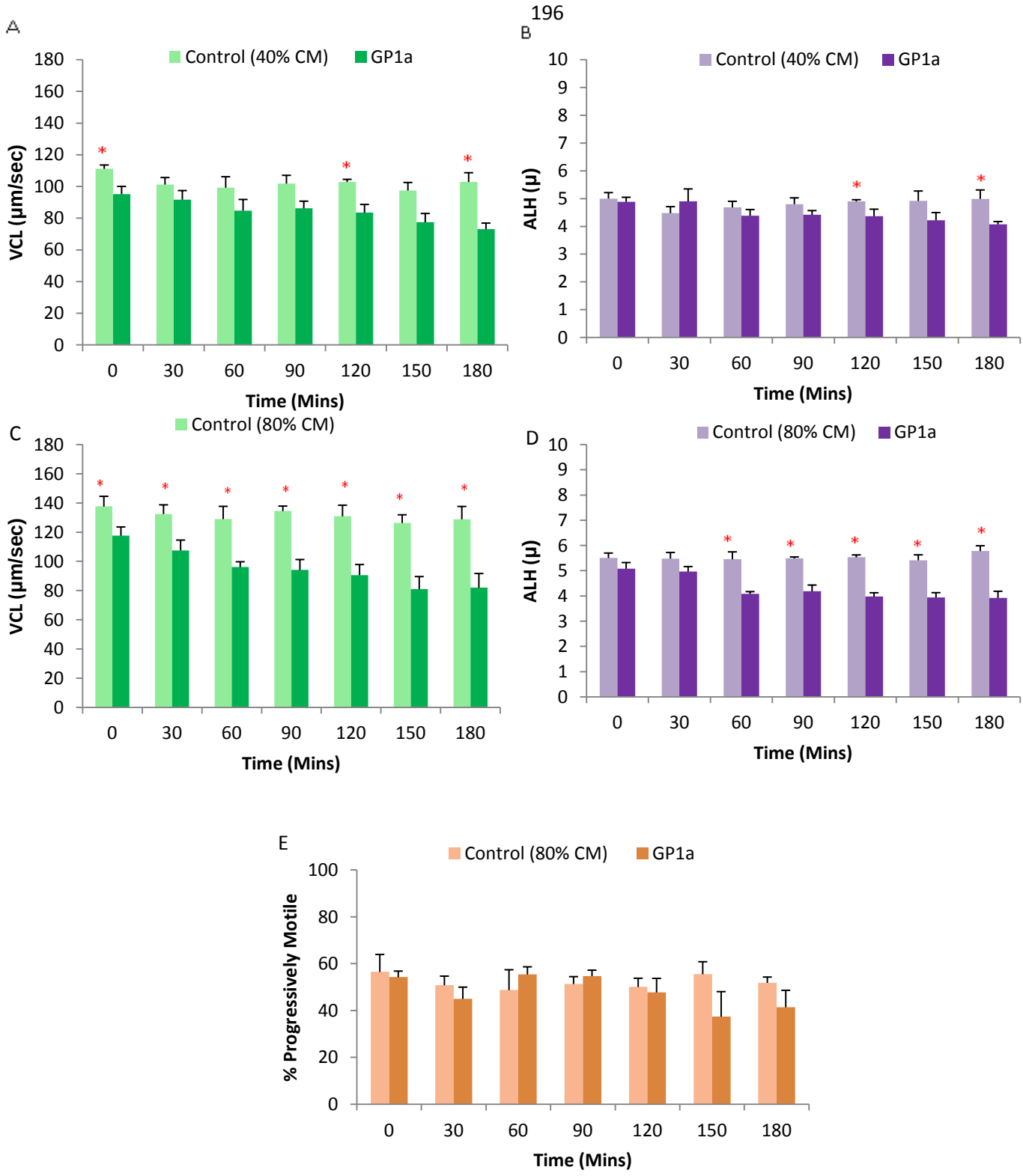


Figure 10-18 Expression of basal (control) motility values compared to treated samples (GP1a) in capacitating (CM) media (40% and 80% fraction). For 40% fraction (A) VCL**, (B)** ALH **and for 80% fraction (C)** VCL, **(D)** ALH and **(E)** % progressive motility. The result shown is the mean ± SE for all parameters. In this study all motility parameters were measured over a period of 180 min under non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

10.9 Treatment with EO1428

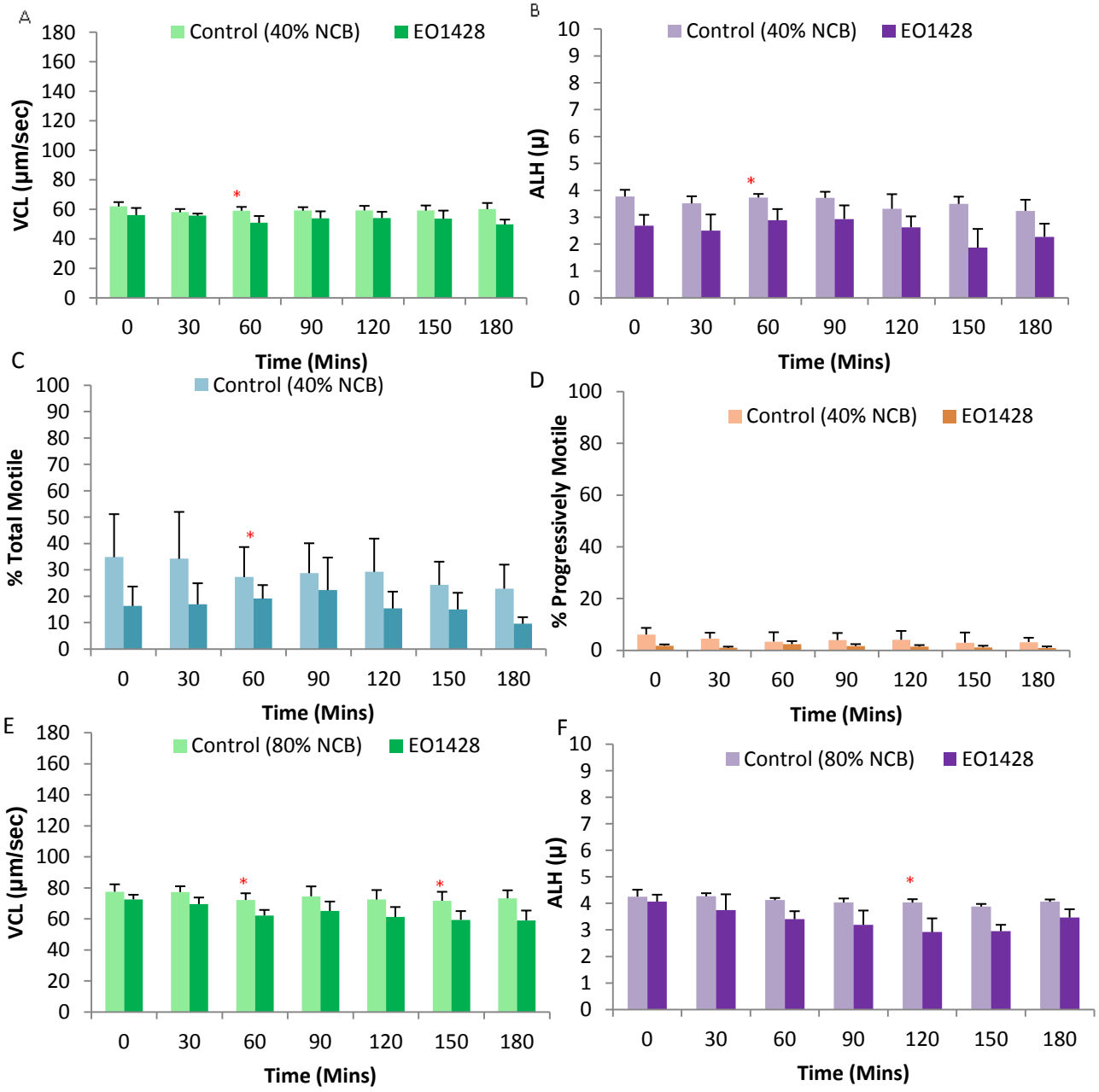


Figure 10-19. Expression of basal (control) motility values compared to treated samples (EO1428) in non-capacitating (NCM) media. Data for 40% fraction under non-capacitating conditions for **(A)** VCL, **(B)** ALH, **(C)** % total motile and **(D)** % progressively motile. Data for 80% fraction; **(E)** VCL and **(F)** ALH. The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 4 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

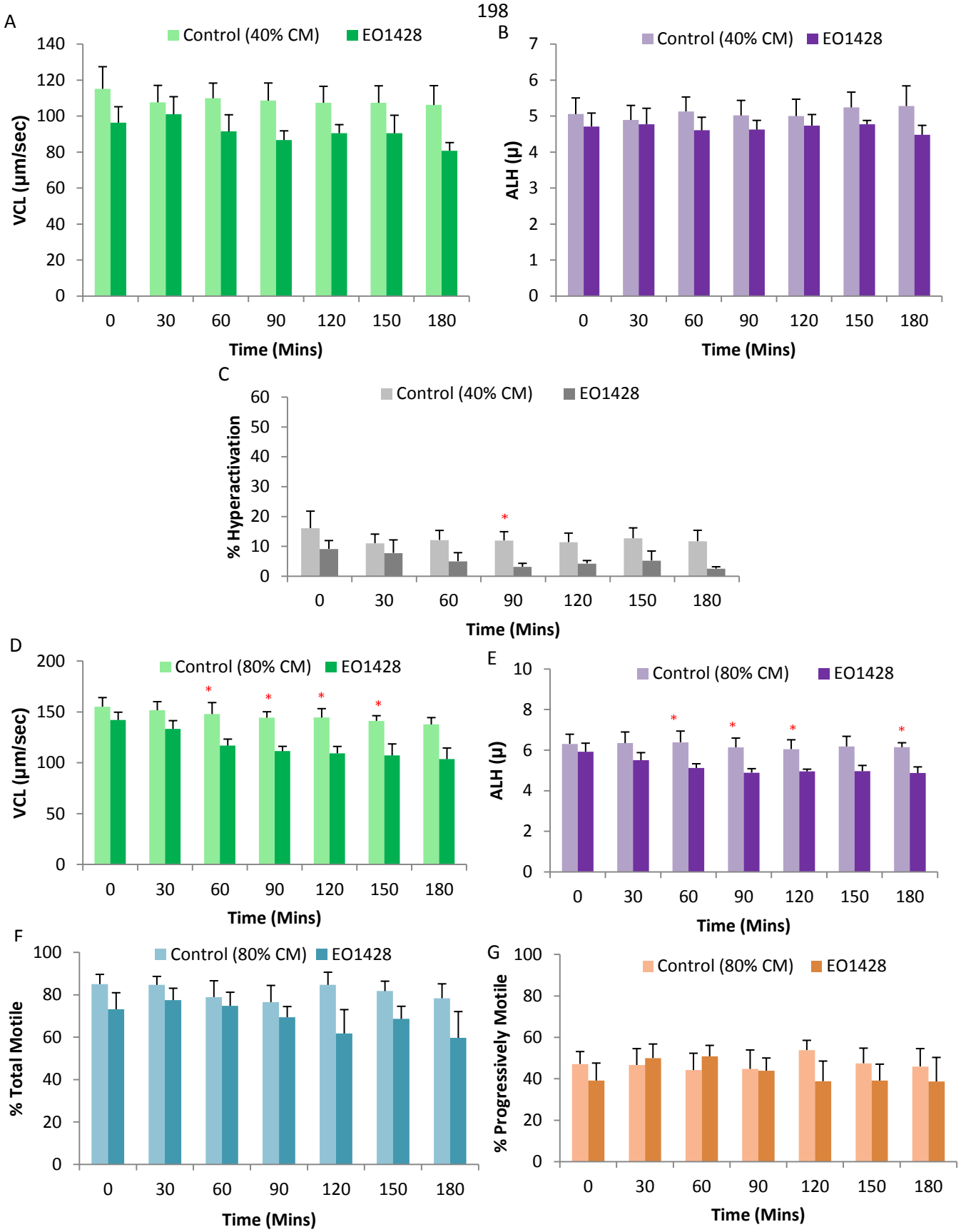


Figure 10-20. Expression of basal (control) motility values compared to treated samples (EO1428) in capacitating (CM) media. Data for 40% fraction for **(A)** VCL, **(B)** ALH, **(C)** % hyperactivation. Data for 80% fraction; **(D)** VCL, **(E)** ALH, **(F)** % total motile and **(G)** % progressively motile. The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 4 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

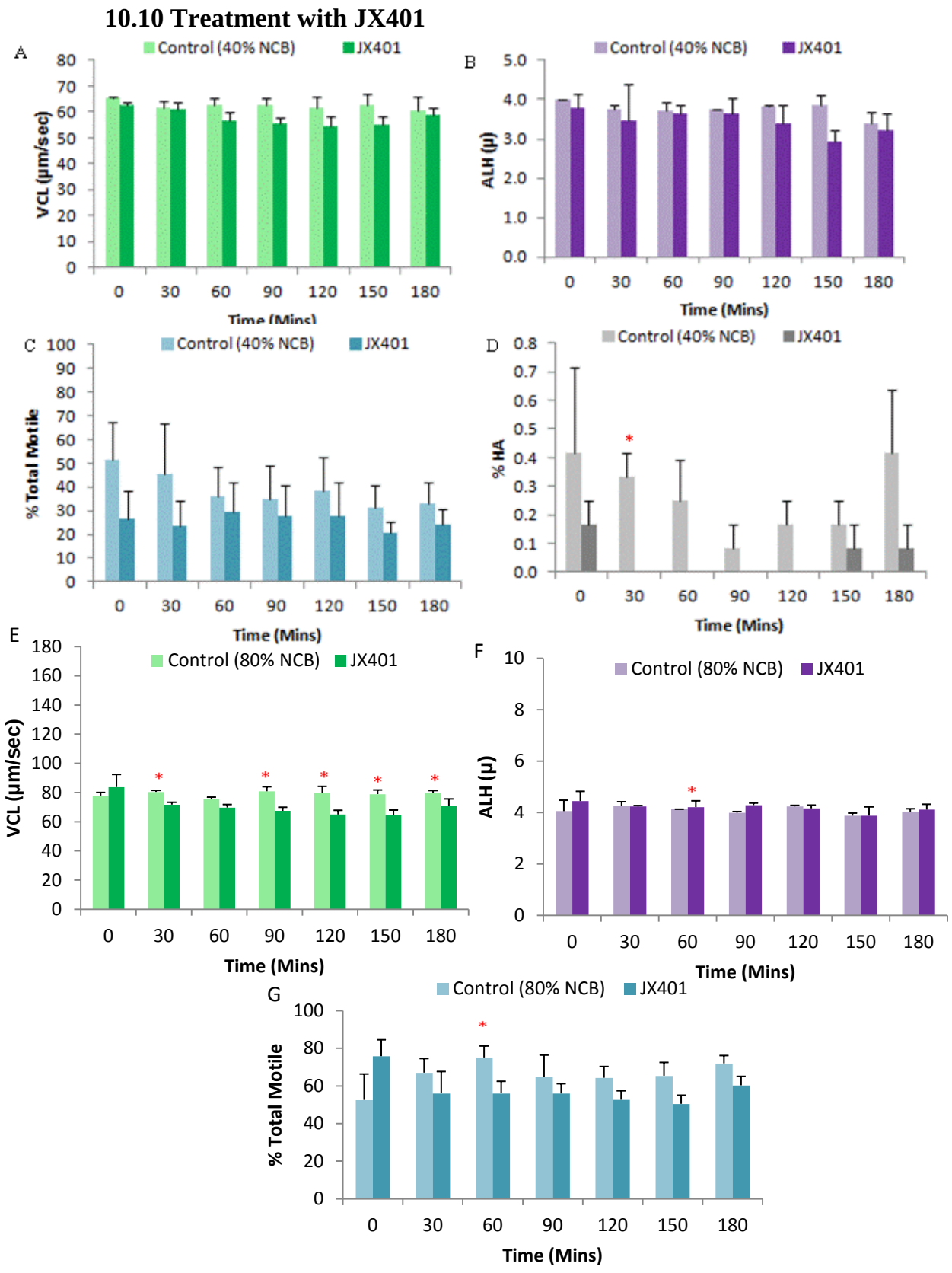


Figure 10-21. Expression of basal (control) motility values compared to treated samples (JX401) in non-capacitating (NCM) media in 40% fractions for (A) VCL, **(B)** ALH, **(C)** % total motile, **(D)** hyperactivation **and 80% fractions (E)** VCL, **(F)** ALH and **(G)** % total motile**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

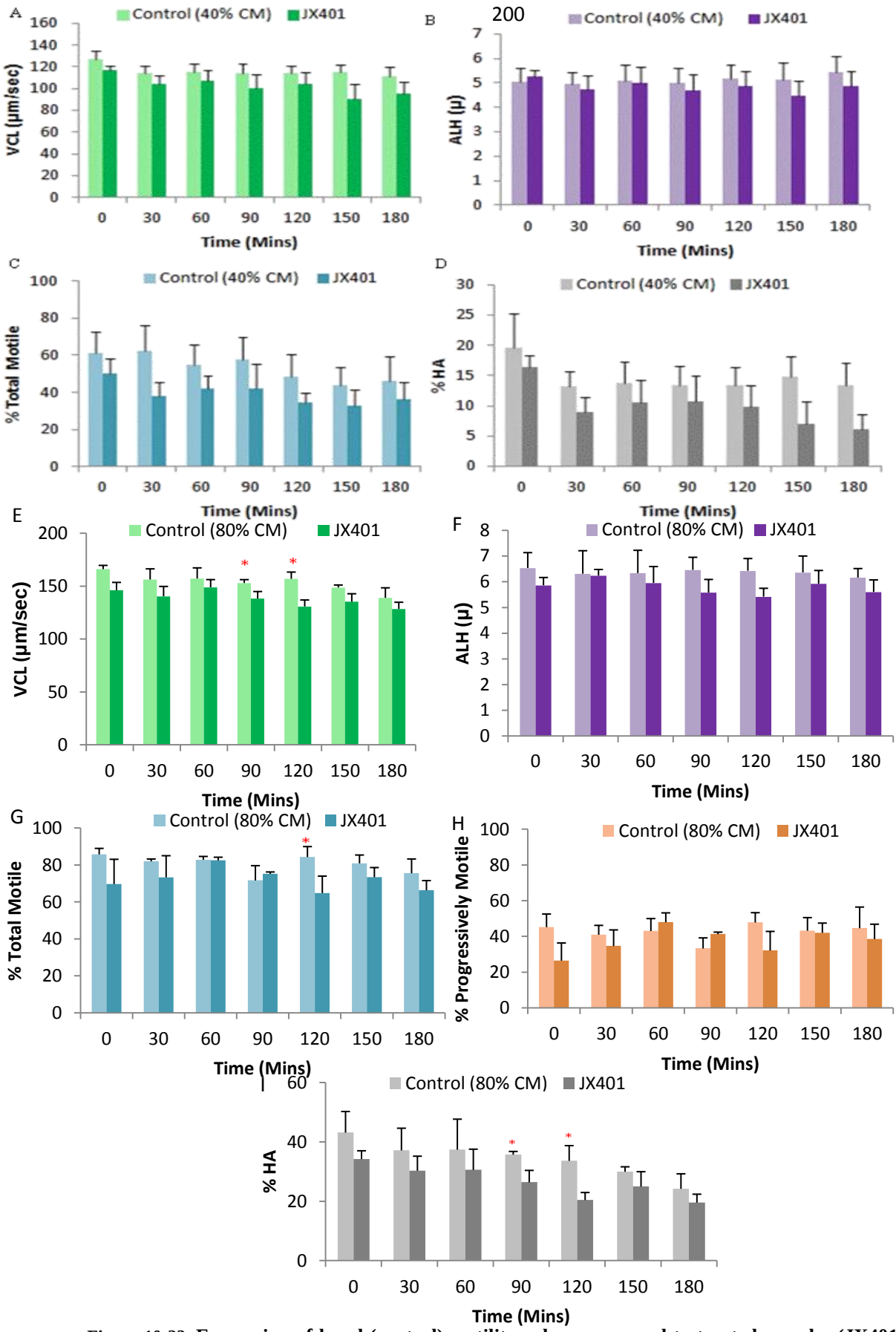


Figure 10-22. Expression of basal (control) motility values compared to treated samples (JX401) in capacitating (CM) media in 40% fractions for (A) VCL, **(B)** ALH, **(C)** % total motile, **(D)** hyperactivation **and 80% fractions (E)** VCL, **(F)** ALH, **(G)** % total motile, **(H)** % progressively motile and **(I)** hyperactivation**.** In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for both parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points.

10.11 Treatment with PHA665752

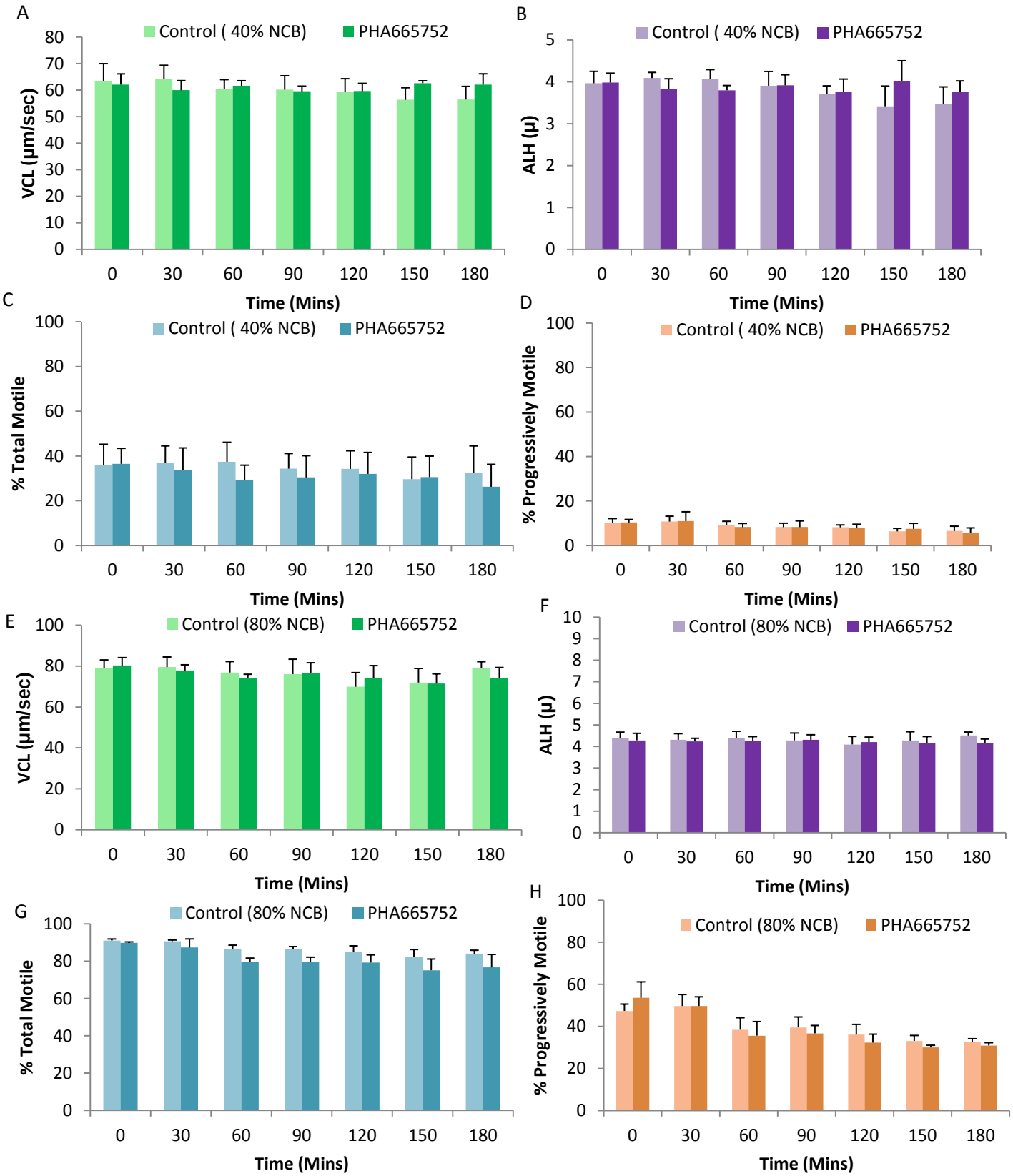


Figure 10-23. Expression of basal (control) motility values compared to treated samples (PHA665752) in non-capacitating (NCM) media in 40% fraction for (A) VCL, **(B)** ALH, **(C)** % total motile, **(D)** % progressive motility and **80% fraction for (E)** VCL, **(F)** ALH, **(G)** % total motile and **(H)** % progressively motile. The result shown is the mean ± SE for motility parameters measured. All motility parameters were measured over a period of 180 min under non-capacitating conditions. N= 3 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points.

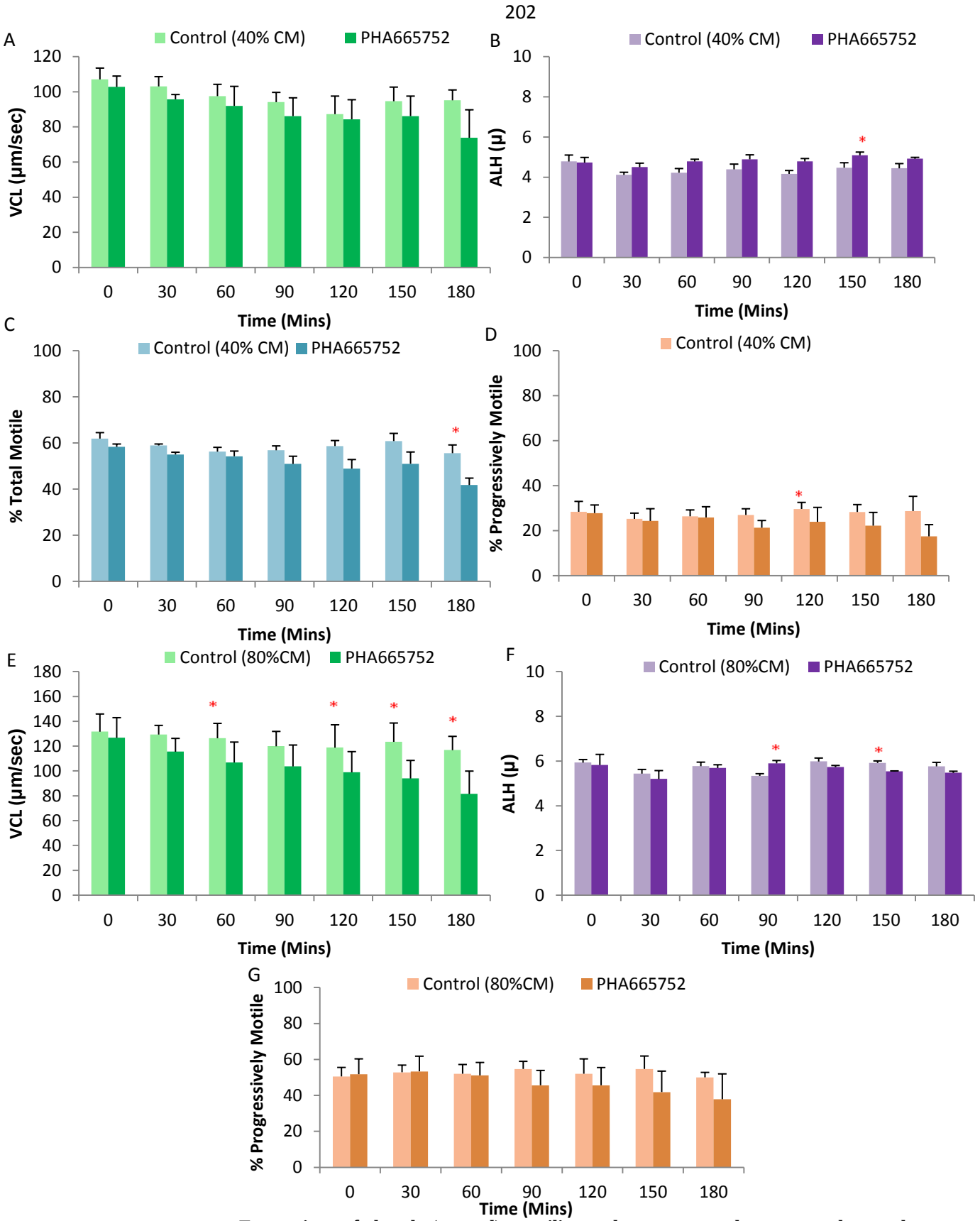


Figure 10-24. Expression of basal (control) motility values compared to treated samples (PHA665752) in capacitating (CM) media in 40% fraction for (A) VCL, **(B)** ALH, **(C)** % total motile and **(D)** % progressively motile **and 80% fraction (E)** VCL, **(F)** ALH and **(G)** % progressively motile**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 3 for all parameters, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

10.12 Treatment with Trequinsin Hydrochloride

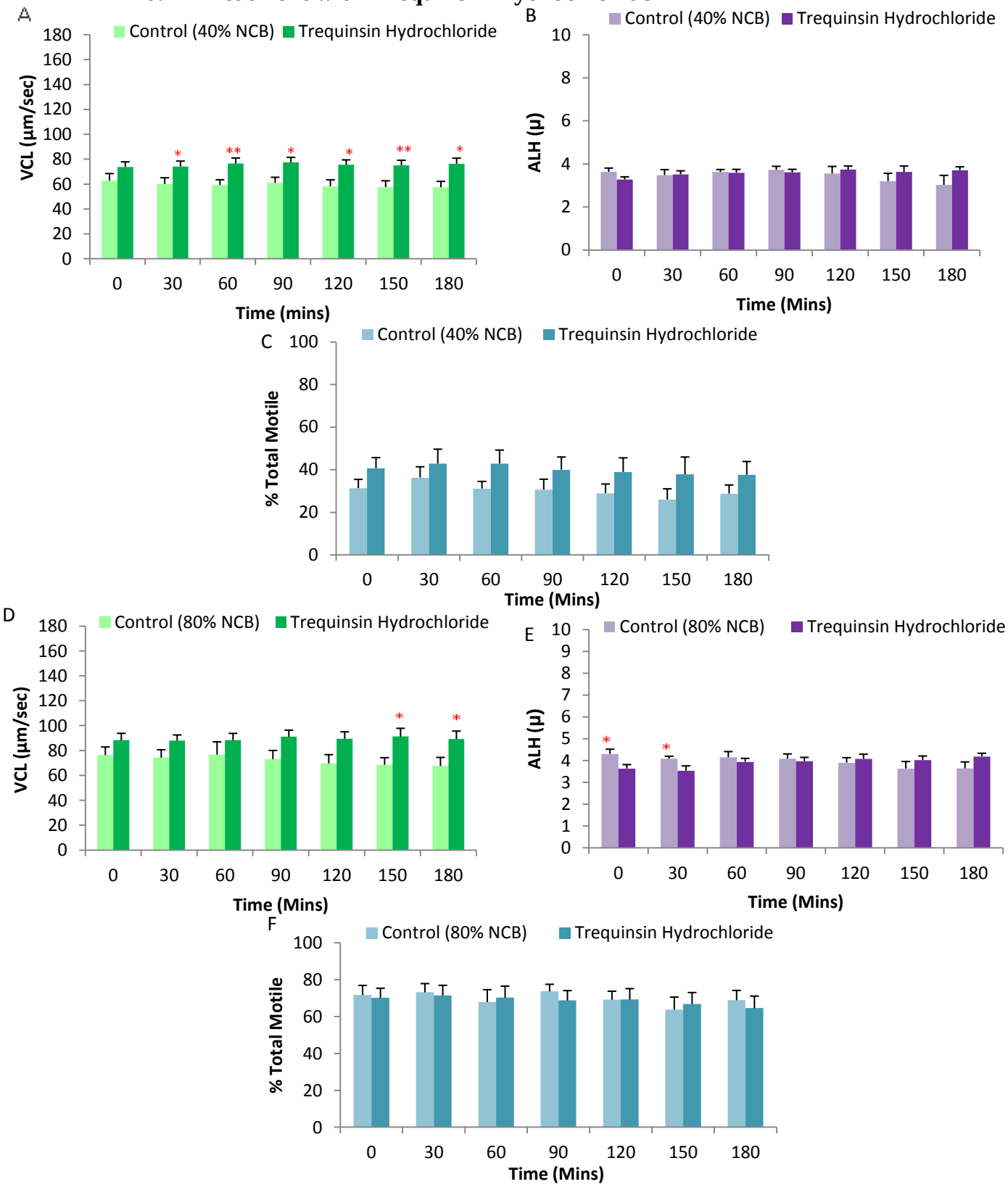


Figure 10-25 Expression of basal (control) motility values compared to treated samples (Trequinsin Hydrochloride) in non-capacitating (NCM) media for 40% fraction (A) ALH, **(B)** VCL and **(C)** % total motile **and 80% fraction (D)** VCL, **(E)** ALH and **(F)** % total motile. The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min under either non-capacitating or capacitating conditions comparing the difference between the control and treated samples. N= 6 for all parameters, * indicates a significant increase (*P<0.05)*, ** indicates P<0.005 between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskall-Wallace test).

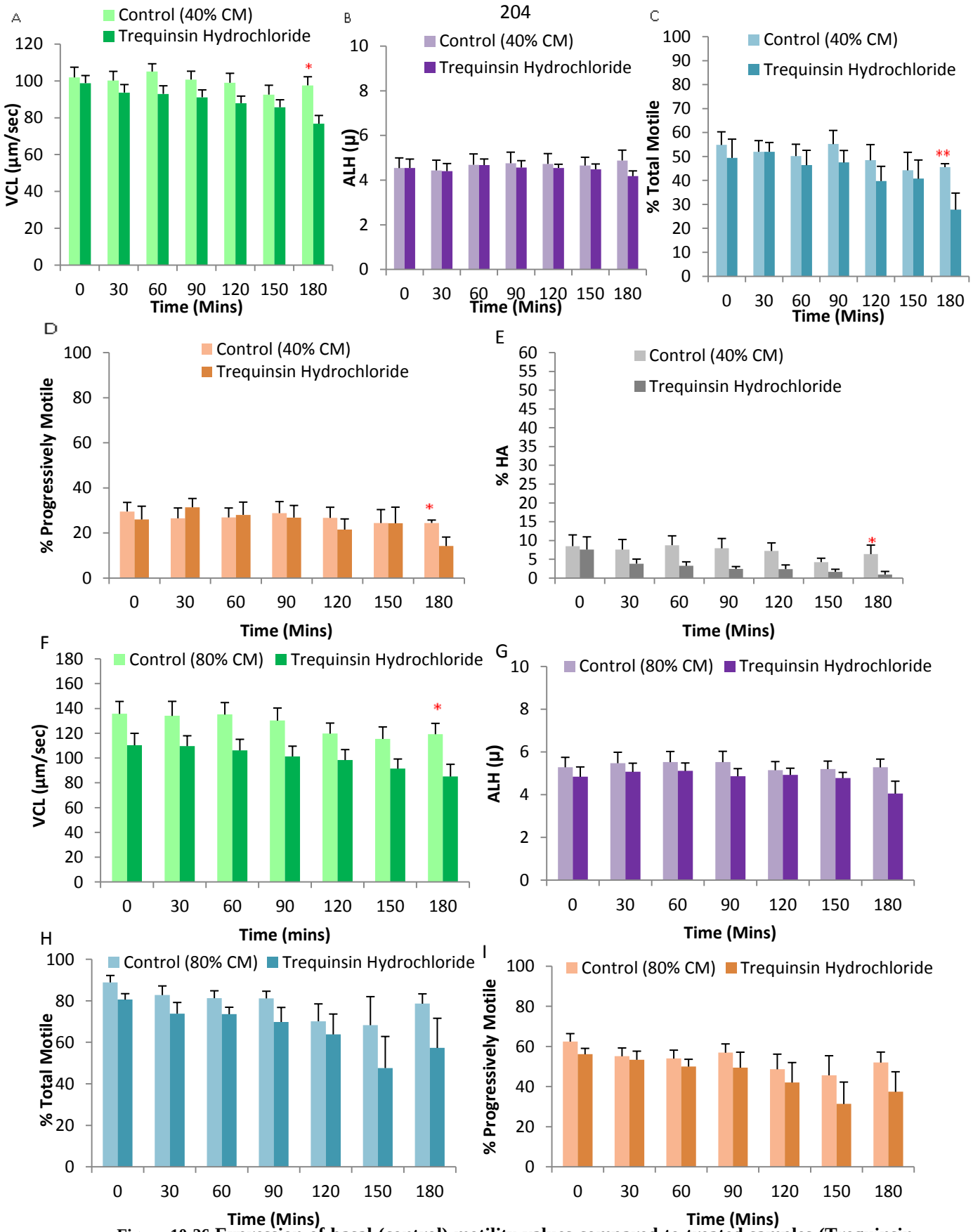


Figure 10-26 Expression of basal (control) motility values compared to treated samples (Trequinsin Hydrochloride) in non-capacitating (NCM) media for 40% fraction (A) VCL, **(B)** ALH, **(C)** % total motile, **(D)** % progressively motile and **(E)** hyperactivation **and 80% fraction (F)** VCL, **(G)** ALH, **(H)** % total motile and **(I)** % progressively motile. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 6 for all parameters, * indicates a significant increase (*P<0.05)*, ** indicates P<0.005 between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskall-Wallace test).

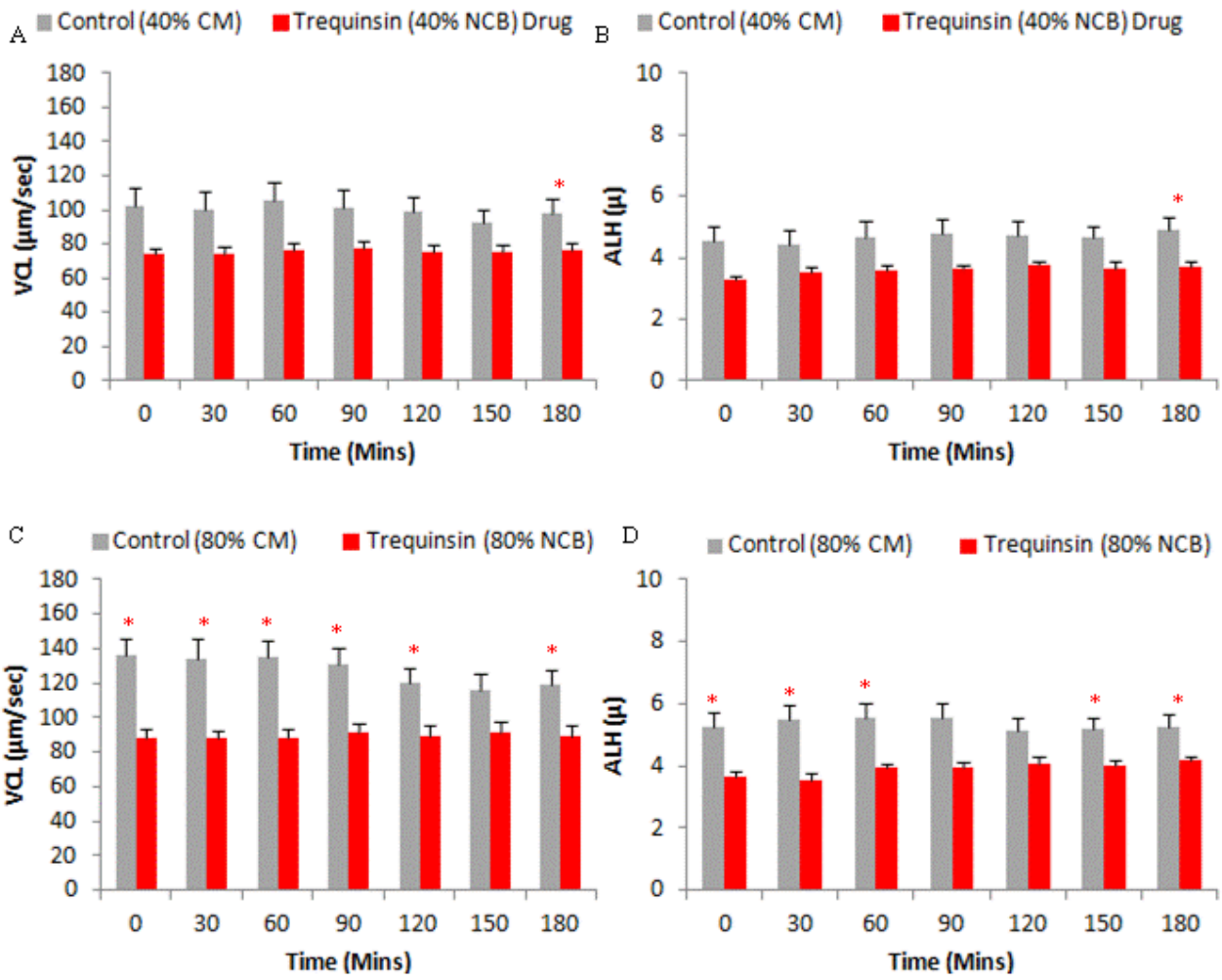


Figure 10-27. Comparing basal (control) motility values under capacitating conditions to Trequinsin treated non-capacitated samples for 40% fraction (A) VCL, **(B)** ALH and **80% fraction (C)** VCL and **(D)** ALH**.** The result shown is the mean ± SE for motility parameters measured. In this study all motility parameters were measured over a period of 180 min. N= 6 for both parameters, * indicates a significant increase (*P<0.05)*, ** indicates P<0.005 between control and treated samples at independent time points (T-test, Mann Whitney test and Kruskall-Wallace test).

10.13 Treatment with Novel Ion Channel Compounds (A1-H1)

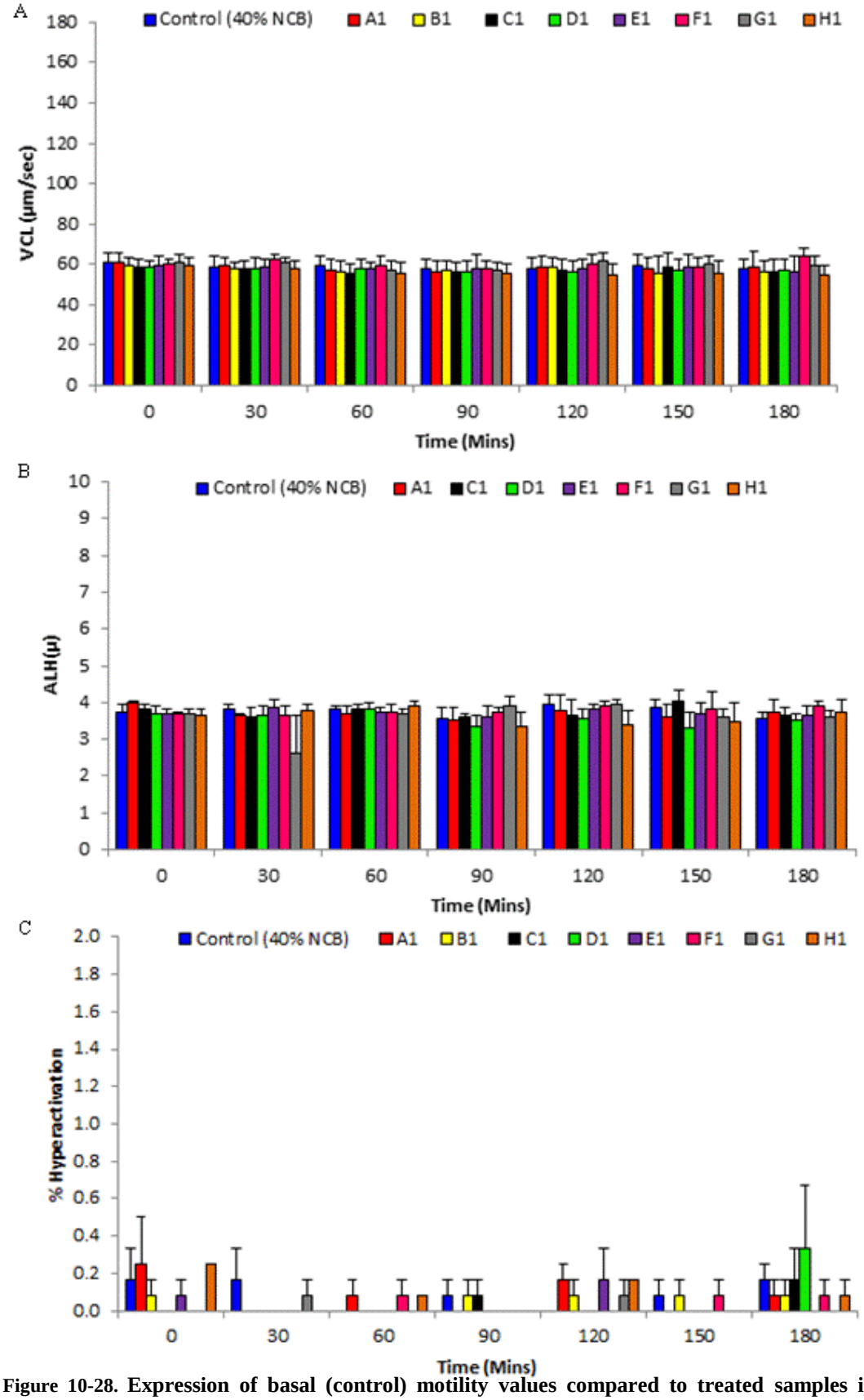


Figure 10-28. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) VCL, **(B)** ALH **(C)** % hyperactivation. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

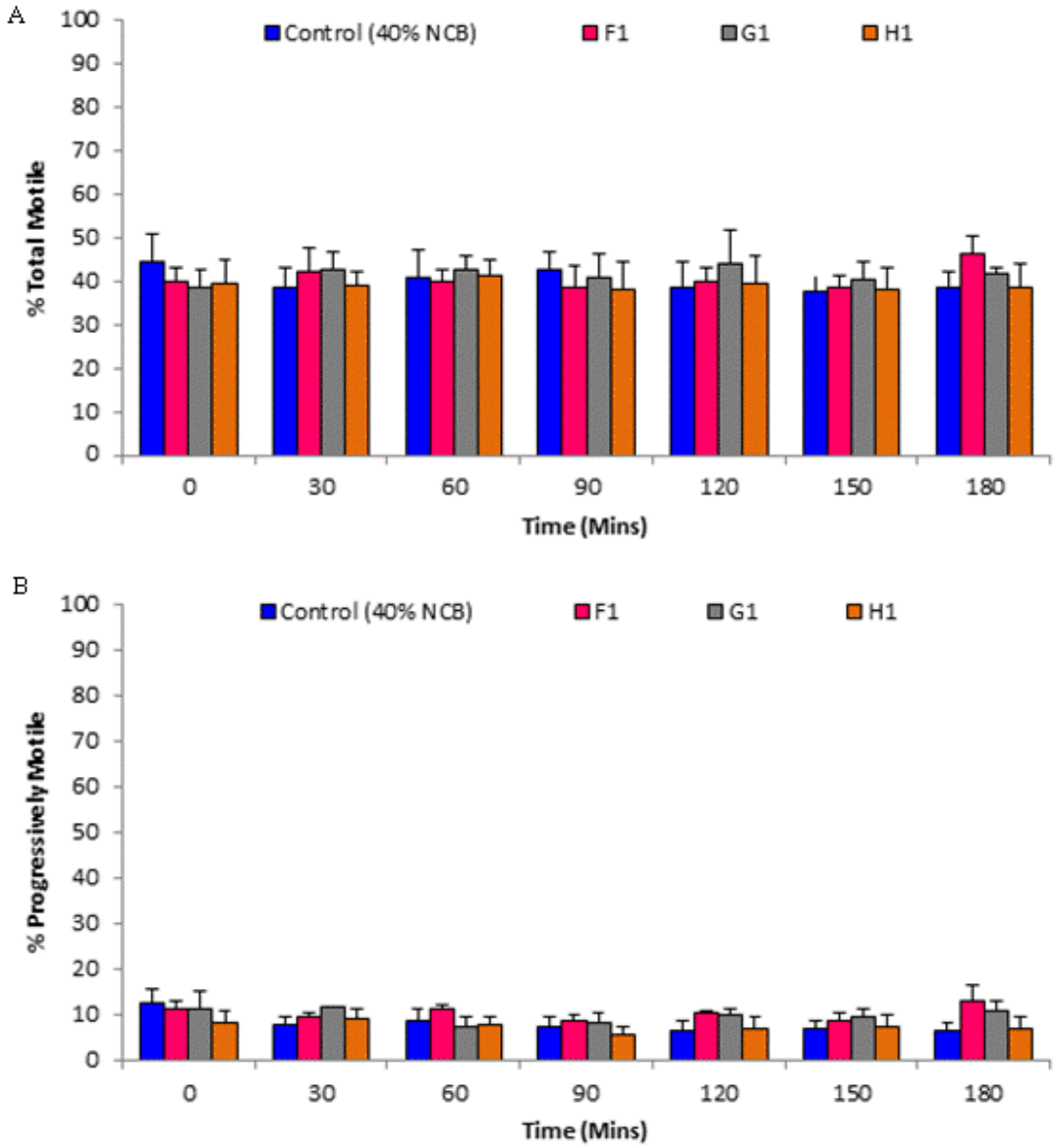


Figure 10-29. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % total motile and **(B)** % progressively motile. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

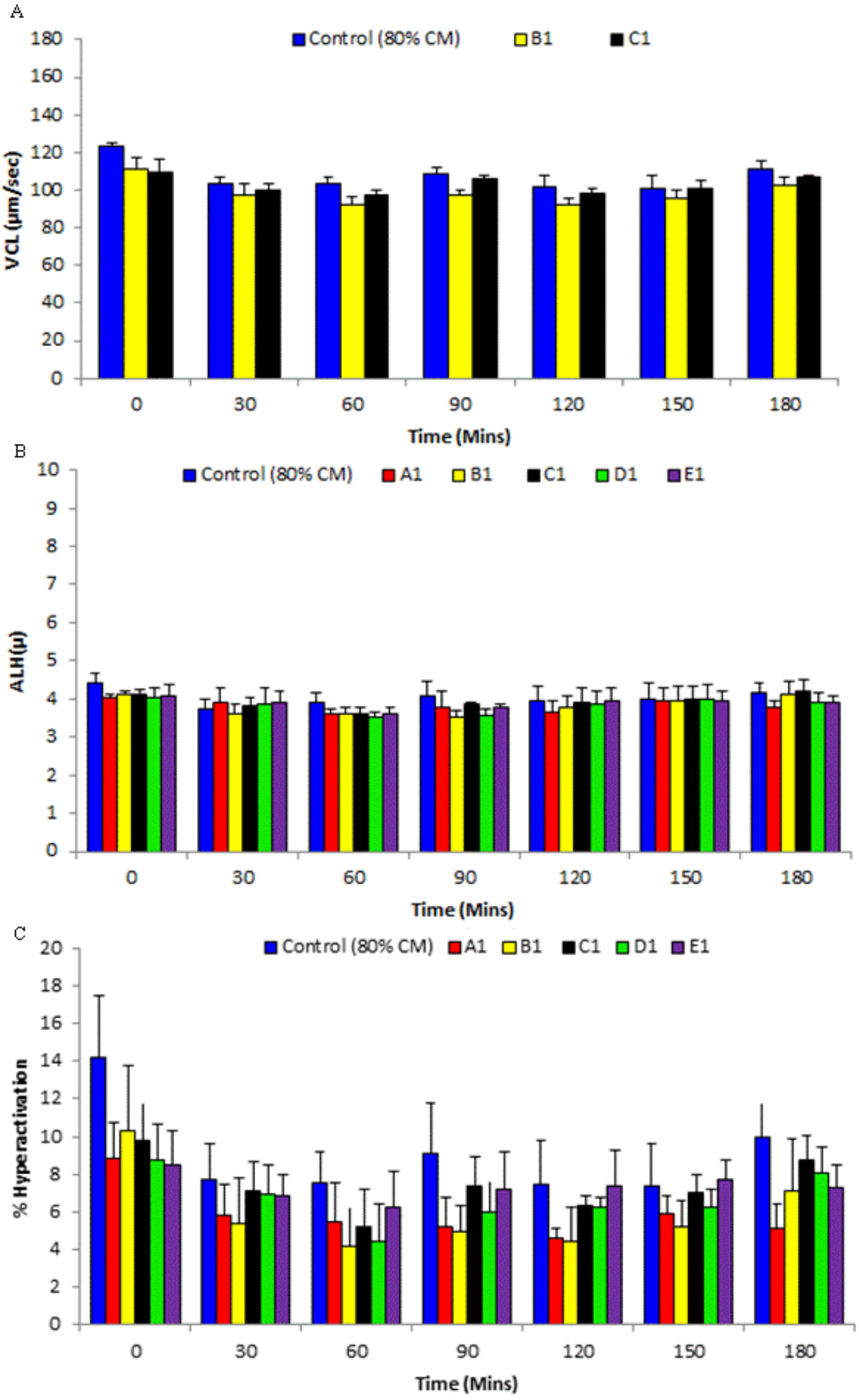


Figure 10-30 Expression of basal (control) motility values compared to treated samples in capacitating media (CM), 80% fraction (A) VCL, **(B)** ALH and **(C)** % hyperactivation. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

10.14 Progesterone Metabolites (Neurosteroids)

10.14.1 Introduction

Progesterone induces hyperactivation, stimulates the acrosome reaction and increases binding and penetrating capabilities of spermatozoa in hamster oocytes (Uhler et al., 1992, Calogero et al., 1996, Bronson et al., 1999, Sueldo et al., 1993). The physiological relevance of this has been proven through sperm incubation with follicular fluid which controls sperm function in relation to the steroid content. When steroids were removed from the follicular fluid the related sperm functional effects (hyperactivation and the acrosome reaction) disappeared (Calogero et al., 2000). Progesterone has been found to significantly increase the number of hyperactivated spermatozoa and is capable of generating an almost three-fold increase in the percentage of hyperactivated sperm in a sample even at low concentrations (Uhler et al., 1992, Calogero et al., 2000, Calogero et al., 1996). A large number of studies have shown that progesterone is capable of modulating sperm function by interacting with a membrane binding site similar to the neuronal γ-aminobutyric acid ($GABA_A$) receptor (Calogero et al., 2000). Activation of the neuronal $GABA_A$ receptor results in an influx of chloride ions through open chloride channels leading to hyperpolarization in the neuron. Progesterone and its metabolites have been implicated in the interaction of GABA with its $GABA_A$ receptor in the central nervous system (Calogero et al., 2000, Lan et al., 1991, Majewska et al., 1986). The presence of GABA in human semen and specifically the identification of $GABA_A$ binding sites on human sperm plasma membranes had not been elucidated until 1995 however in 1993 Wistrom and Meizel observed the involvement of a putative GABAergic receptor in the progesterone induced acrosome reaction (Aurell and Meizel, 1993, Aanesen et al., 1995). However, the $GABA_A$ receptor identified on sperm is different to the neuronal $GABA_A$ receptor and results in an efflux of chloride ions rather than an influx as seen in neuronal tissue (Aurell and Meizel, 1993). The presence of $GABA_A$ receptors and their biological competences has

been substantiated by a numerous studies and it has been shown that GABA independently is capable of initiating the acrosome reaction in human sperm (Aurell and Meizel, 1993, Aanesen et al., 1995, Ritta et al., 1998). Calogero *et al* identified that GABA is also able to modulate sperm kinematic parameters in normal men and this modulation involved the $GABA_A$ receptor specifically (Calogero et al., 1996). However, the effect of GABA on sperm motility is still controversial with Aanesen *et al* not being able to identify any effect of GABA on sperm motility (Aanesen et al., 1995).

The Dundee drug discovery unit had previously produced a library similar to the Chemogenomics library (chapter 5 and 6) which consisted of neurosteroids which are metabolites of progesterone. These compounds are also allosteric modulators of $GABA_A$ receptors which included the $GABA_A$ agonist muscimol and the modulator diazepam along with DHEA, 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one and DHEA sulphate. From the knowledge of the effect of GABA and progesterone on sperm function via the $GABA_A$ receptor it was decided to include some compounds from this library in the motility assessment. These compounds were evaluated identically to the compounds from the Chemogenomics library and were selected for their ability to increase intracellular Ca^{2+}.

10.14.2 Results

Prepared sperm cells were collected from the 40% fraction after density gradient centrifugation and placed in appropriate incubators under non-capacitating conditions. Following this, cells were treated with the appropriate compound and motility analysis conducted on addition of compound and then at 30 min intervals over a period of 3 hrs (see chapter 2, methods).

No significant changes were identified from treatment at any time point, in any of the kinematic parameters assessed (VCL, ALH, total motile and progressively motile) for DHEA, 5β-pregnan-3α-ol-20-one and diazepam (figures 10-31 A-C and 10-32 A). However, muscimol and 5α-pregnan-3α-ol-20-one were found to significantly increase ALH instantaneously (0 min, $P<0.05$, figure 10-31 B) although this effect was not sustained past this point. 5α-pregnan-3α-ol-20-one and DHEA sulphate were then found to significantly decrease ALH after 120 min incubation ($P<0.05$, figure 10-31 B). No other significant changes were noted for these compounds in any other parameter at any other time point.

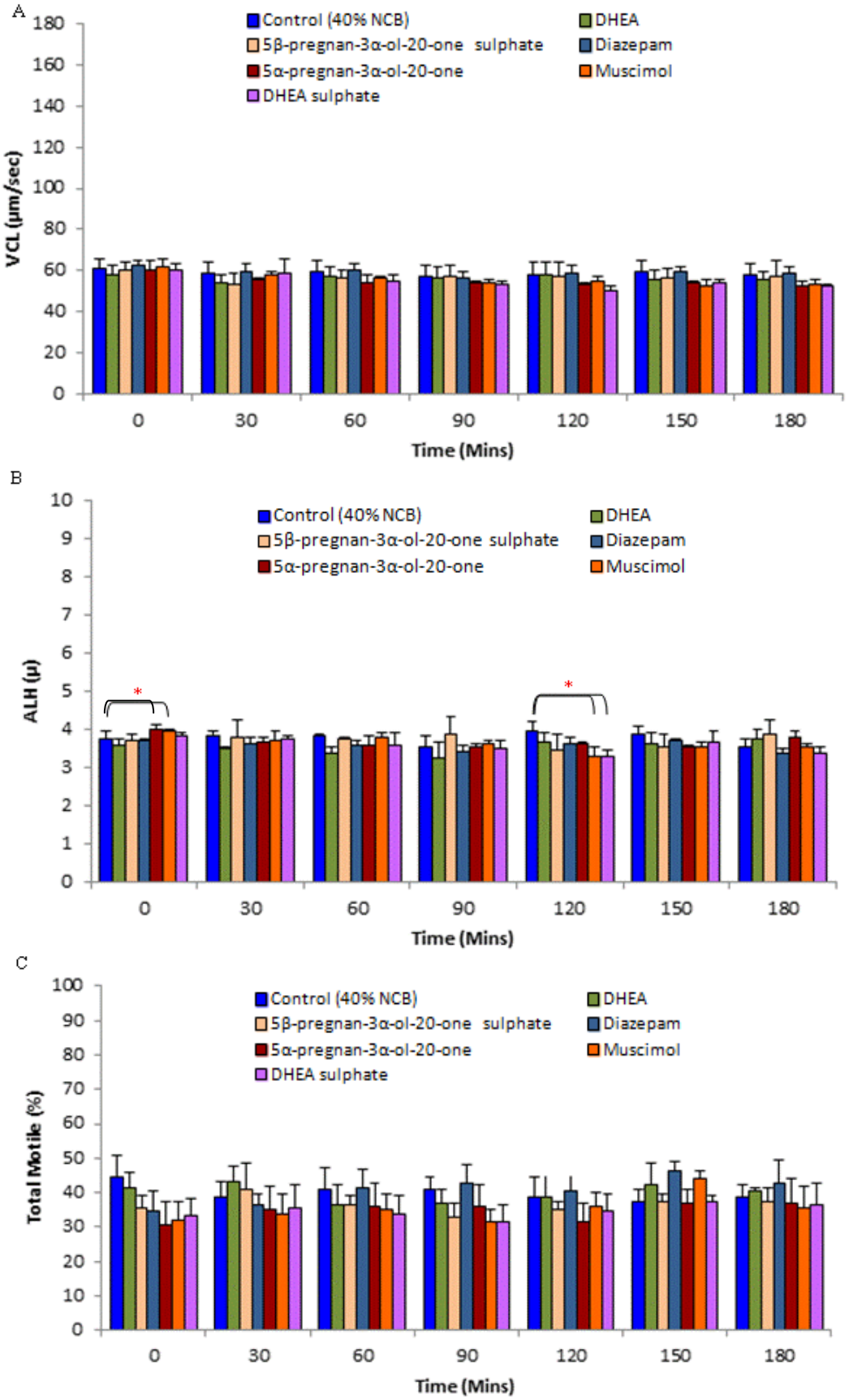


Figure 10-31. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) VCL, **(B)** ALH and **(C)** % total motile. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

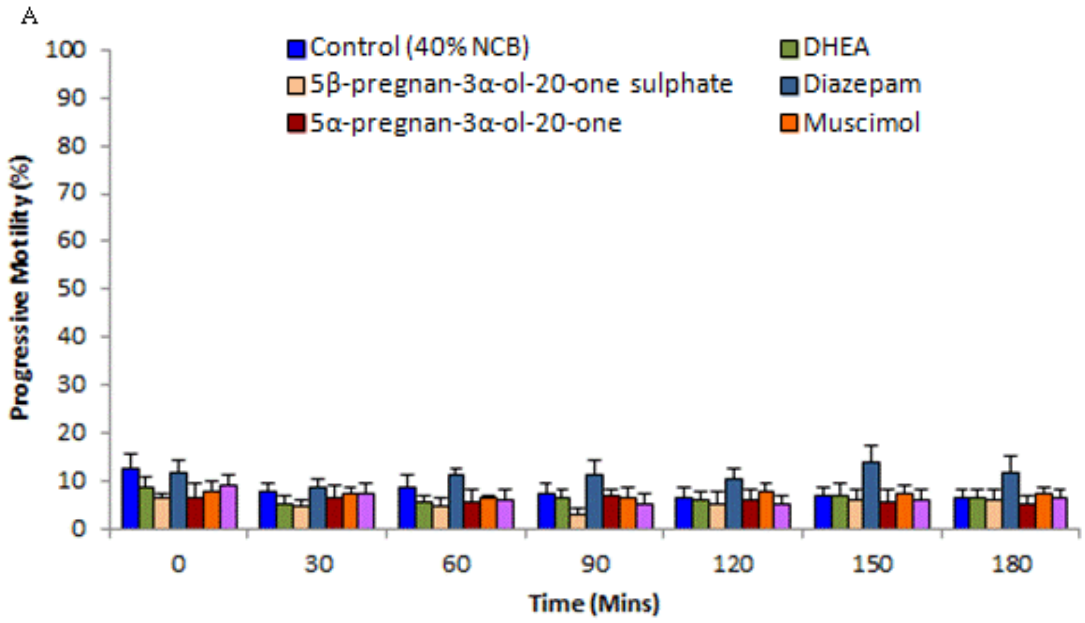


Figure 10-32. Expression of basal (control) motility values compared to treated samples in non-capacitating (NCM) media, 40% fraction (A) % progressive motility. The result shown is the mean ± SE. In this study all motility parameters were measured over a period of 180 min under non-capacitating conditions comparing the difference between the control and treated samples. N= 3 for all compounds, * indicates a significant increase (*P<0.05)* between control and treated samples at independent time points (Mann Whitney test and Kruskall-Wallace test).

10.14.3 Discussion

As mentioned previously the effect of compounds affecting sperm motility through $GABA_A$ receptors is controversial. The results from this study overall corroborate the results from Aanesen *et al* (1995) identifying very few significant changes in motility (Aanesen et al., 1995). However, muscimol was found to stimulate ALH in this study which is similar to the results observed by Calogero *et al* (1996) (Calogero et al., 1996). The study by Calogero *et al* (1996) found treatment with muscimol also increased VCL, the percentage of active sperm and hyperactivation which was not seen in this study (Calogero et al., 1996). Ritta *et al* (1998) also identified significant increases in hyperactivation as a result of muscimol treatment suggesting under the correct conditions this compound has the potential to promote hyperactivated motility (Ritta et al., 1998). The reason for this discrepancy is most likely owing to the difference in experimental protocol with samples in this study being from the 40% fraction under non-capacitating conditions.

Numerous studies have linked compounds that act via $GABA_A$ receptors to the acrosome reaction and a proposed model for this suggests the chloride flux in spermatozoa, plays a role in the regulation of the secondary Ca^{2+} transient, essential for the acrosome reaction (Tesarik, 1996, Aurell and Meizel, 1993, Shi and Roldan, 1995). The chloride induced depolarization which results from binding to the $GABA_A$ receptor is thought to open an additional set of channels that are voltage regulated, leading to a secondary Ca^{2+} transient and the initiation of the acrosome reaction (Tesarik, 1996, Meizel et al., 1997). Meizel *et al* (1997) were the first group to detect a ligand mediated Ca^{2+} wave in sperm and they proposed that the efflux of chloride ions as a result of $GABA_A$ receptor binding of progesterone influences Ca^{2+} influx during the acrosome reaction (Meizel et al., 1997). This Ca^{2+} influx could explain the increased $[Ca^{2+}]_i$ measured by the Flexstation when spermatozoa were treated with these compounds. It

would be beneficial to complete acrosome studies on these compounds to confirm this theory. Further analysis was not conducted on these compounds owing to the minimal changes in motility expressed as a result of incubation with these compounds.

10.15 Clinic A and B datasets

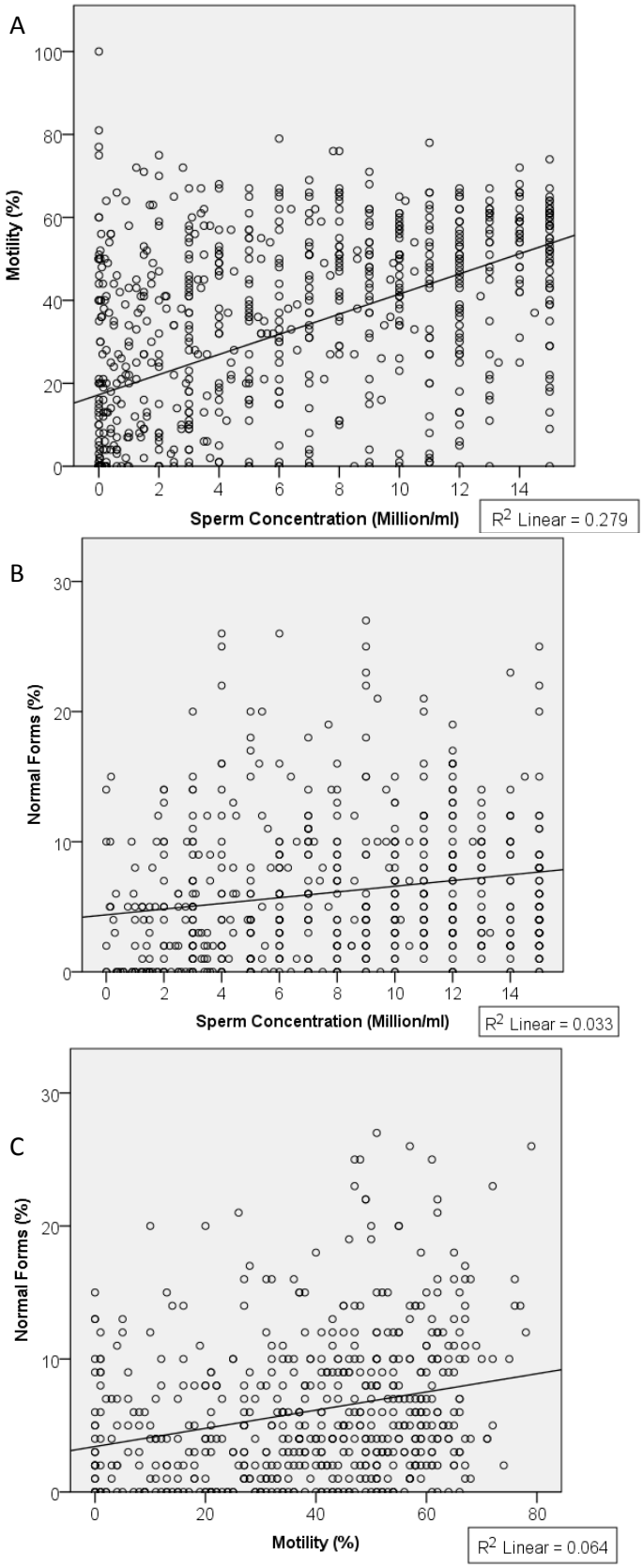


Figure 10-33 Scatter plots identifying correlations for Clinic A for the lower limit group (≤15 million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).

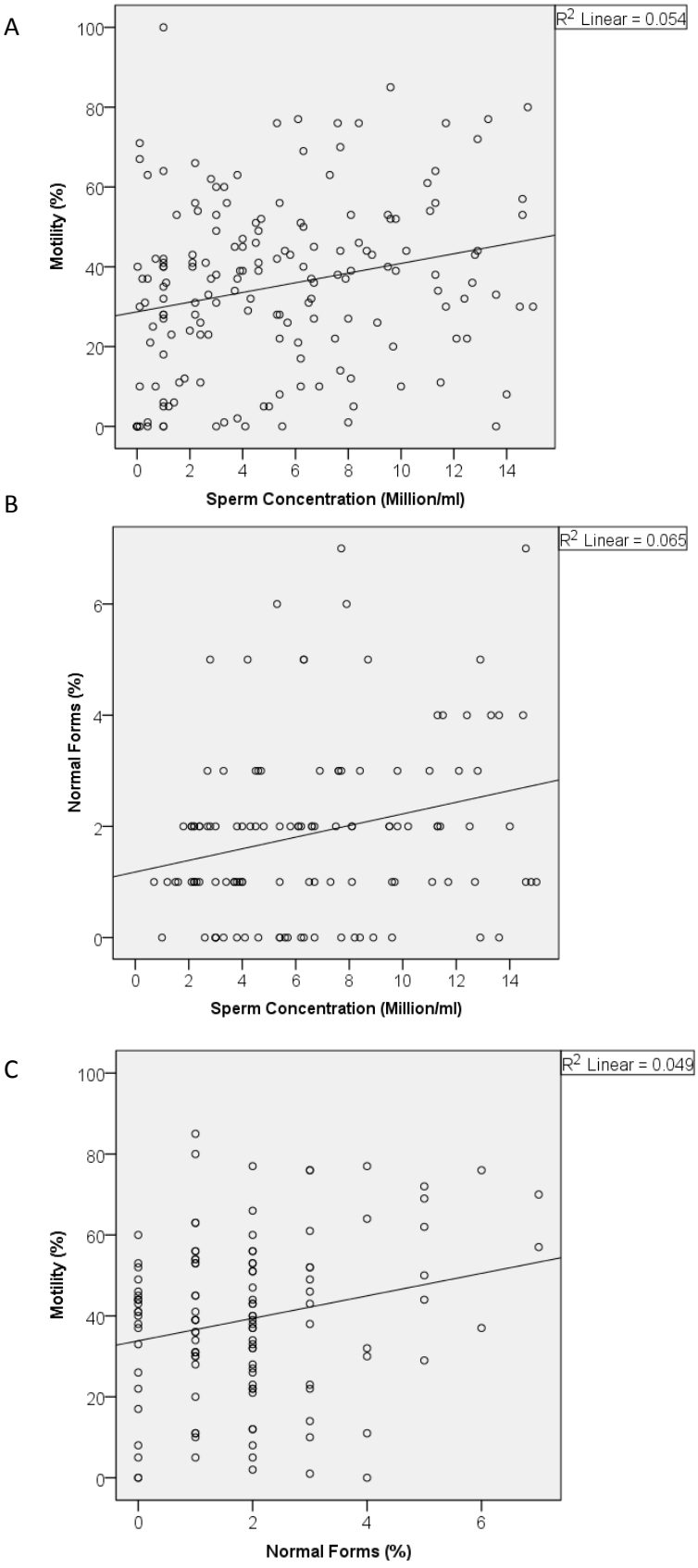


Figure 10-34 Scatter plots identifying correlations for Clinic B for the lower limit group (≤15 million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).

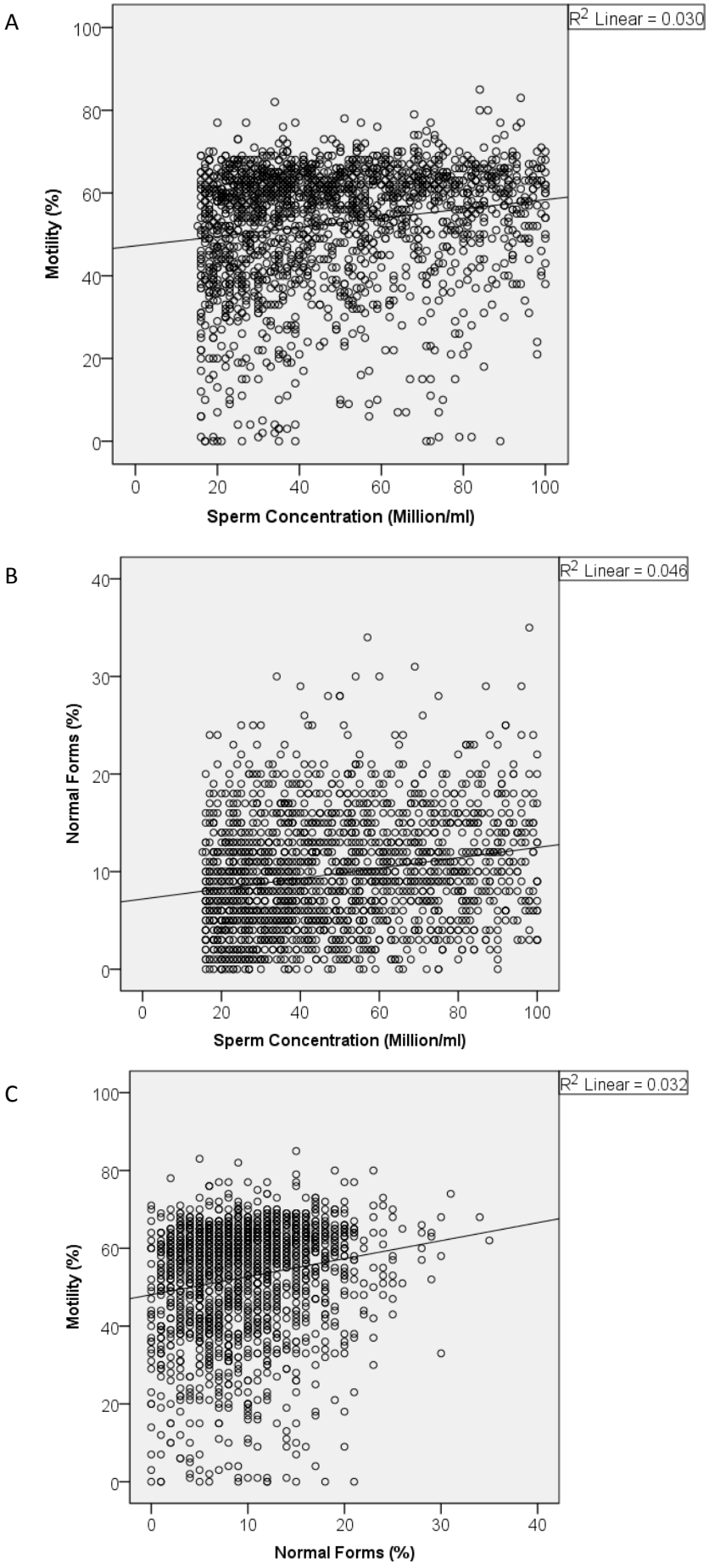


Figure 10-35 Scatter plots identifying correlations for Clinic A for the upper limit group (≤100 million/ml).2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).

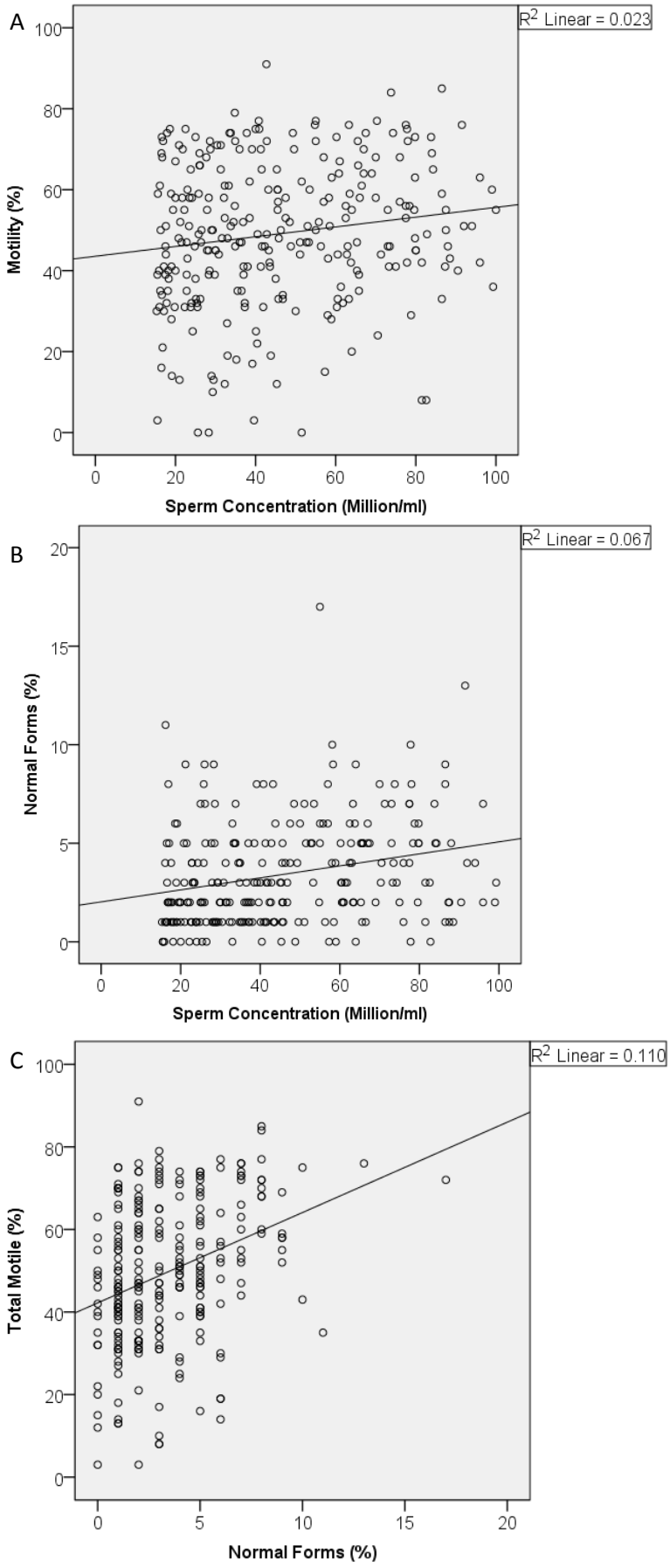


Figure 10-36 Scatter plots identifying correlations for Clinic B for the upper limit group (≤100 million/ml). 2-D plots looking at correlations between motility and morphology (A), motility and concentration (B) and morphology and concentration (C).

10.16 Consent form for patient/donor participation in research

ASSISTED CONCEPTION UNIT

WARD 35

NHS TAYSIDE

NINEWELLS HOSPITAL

DUNDEE DD1 9SY

Direct line (01382) 632111

Fax (01382) 633853

CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

Title of research: Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVF success.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed…….

Name (block capitals)……..

Date……

Witnessed……(name)……Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, e.barratt@dundee.ac.uk

ASSISTED CONCEPTION UNIT
WARD 35

NHS TAYSIDE
NINEWELLS HOSPITAL
DUNDEE DD1 9SY

Patient/donor consent form

Research to improve our understanding of how human sperm function

I have read the research study information sheet and have had the opportunity to ask questions YES / NO

I understand that I can withdraw consent or change my mind at any time YES / NO

Whether I participate or not, I understand that this will have no impact on my treatment or care either now or in the future YES / NO

I consent to allowing researchers to use left-over semen following diagnosis or treatment YES / NO

I am prepared to be contacted for a further sample, if required YES / NO

If yes: contact details

SIGNED……..

NAME (BLOCK CAPITALS)……

DATE……

WITNESSED……(NAME)……(SIGNATURE)

If you have any further questions or queries you can contact either nurse Evelyn Barratt (07528 558624 or email e.barratt@dundee.ac.uk) or Dr Sarah Martins da Silva (01382 660111 bleep 4535 or email s.martinsdasilva@dundee.ac.uk)

References

AANESEN, A., FRIED, G., ANDERSSON, E. & GOTTLIEB, C. 1995. Molecular Human Reproduction: Evidence for γ-aminobutyric acid specific binding sites on human spermatozoa. *Human Reproduction,* 10**,** 1885-1890.

ABU-HEIJA, A. T., FLEMING, R., JAMIESON, M. E., YATES, R. W. S. & COUTTS, J. R. T. 1996. The Effect of Sperm-Mucus Interaction Test on the Outcome of in vitro Fertilization and Ovulation Induction Combined with Intrauterin Insemination. *Journal of Obstetrics and Gynaecology Research,* 22**,** 229-233.

ADAMS, R. H., PORRAS, A., ALONSO, G., JONES, M., VINTERSTEN, K., PANELLI, S., VALLADARES, A., PEREZ, L., KLEIN, R. & NEBREDA, A. R. 2000. Essential Role of p38± MAP Kinase in Placental but Not Embryonic Cardiovascular Development. *Molecular cell,* 6**,** 109-116.

AGARWAL, A. & SAID, T. 2011. Interpretation of Basic Semen Analysis and Advanced Semen Testing. *In:* SABANEGH, E. S. (ed.) *Male Infertility.* Humana Press.

AGIRREGOITIA, E., CARRACEDO, A., SUBIRÁN, N., VALDIVIA, A., AGIRREGOITIA, N., PERALTA, L., VELASCO, G. & IRAZUSTA, J. 2010a. The CB2 cannabinoid receptor regulates human sperm cell motility. *Fertility and sterility,* 93**,** 1378-1387.

AGIRREGOITIA, E., CARRACEDO, A., SUBIRÁN, N., VALDIVIA, A., AGIRREGOITIA, N., PERALTA, L., VELASCO, G. & IRAZUSTA, J. 2010b. The CB(2) cannabinoid receptor regulates human sperm cell motility. *Fertility and sterility,* 93**,** 1378-87.

AITKEN, R. J. 2006. Sperm function tests and fertility. *International Journal of Andrology,* 29**,** 69-75.

AITKEN, R. J., BOWIE, H., BUCKINGHAM, D., HARKISS, D., RICHARDSON, D. W. & WEST, K. M. 1992. Sperm Penetration into a Hyaluronic Acid Polymer as a Means of Monitoring Functional Competence. *Journal of Andrology,* 13**,** 44-54.

AITKEN, R. J., BUCKINGHAM, D. W., BRINDLE, J., GOMEZ, E., BAKER, H. W. & IRVINE, D. S. 1995. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Human reproduction (Oxford, England),* 10**,** 2061-71.

AITKEN, R. J., BUCKINGHAM, D. W. & FANG, H. G. 1993. Analysis of the Responses of Human Spermatozoa to A23187 Employing a Novel Technique for Assessing the Acrosome Reaction. *Journal of Andrology,* 14**,** 132-141.

ALASMARI, W. 2013. *Regulation of calcium signalling for flagellar activity in human sperm and its significance in male fertility.* phD, University of Dundee.

ALASMARI, W., BARRATT, C. L. R., PUBLICOVER, S. J., WHALLEY, K. M., FOSTER, E., KAY, V., MARTINS DA SILVA, S. & OXENHAM, S. K. 2013a. The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation. *Human reproduction (Oxford, England),* 28**,** 866-876.

ALASMARI, W., COSTELLO, S., CORREIA, J., OXENHAM, S. K., MORRIS, J., FERNANDES, L., RAMALHO-SANTOS, J., KIRKMAN-BROWN, J., MICHELANGELI, F., PUBLICOVER, S. & BARRATT, C. L. R. 2013b. Ca2+ Signals Generated by CatSper and Ca2+ Stores Regulate Different Behaviors in Human Sperm. *Journal of Biological Chemistry,* 288**,** 6248-6258.

ALMOG, T., LAZAR, S., REISS, N., ETKOVITZ, N., MILCH, E., RAHAMIM, N., DOBKIN-BEKMAN, M., ROTEM, R., KALINA, M., RAMON, J., RAZIEL, A., BRIETBART, H., SEGER, R. & NAOR, Z. 2008. Identification of Extracellular Signal-regulated Kinase 1/2 and p38 MAPK as Regulators of Human Sperm Motility and Acrosome Reaction and as Predictors of Poor Spermatozoan Quality. *Journal of Biological Chemistry,* 283**,** 14479-14489.

ALMOG, T. & NAOR, Z. 2008. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. *Molecular and Cellular Endocrinology,* 282**,** 39-44.

ALMOG, T. & NAOR, Z. 2010. The role of Mitogen activated protein kinase (MAPK) in sperm functions. *Molecular and Cellular Endocrinology,* 314**,** 239-243.

ALSENZ, J. & KANSY, M. 2007. High throughput solubility measurement in drug discovery and development. *Advanced drug delivery reviews,* 59**,** 546-67.

ALVAREZ, C. 2003. Biological variation of seminal parameters in healthy subjects. *Human Reproduction,* 18**,** 2082-2088.

AMDANI, S. N., JONES, C. & COWARD, K. 2013. Phospholipase C zeta (PLCζ): Oocyte activation and clinical links to male factor infertility. *Advances in Biological Regulation,* 53**,** 292-308.

AMOAKO, A. A., GEBEH, A. K., MARCZYLO, E. L., WILLETS, J. M., ELSON, J., MARCZYLO, T. H. & KONJE, J. C. 2013a. Impact of reference gene selection for type 2 cannabinoid receptor gene expression studies in human spermatozoa. *Andrologia,* 45**,** 278-284.

AMOAKO, A. A., MARCZYLO, T. H., LAM, P. M. W., WILLETS, J. M., DERRY, A., ELSON, J. & KONJE, J. C. 2010. Quantitative analysis of anandamide and related acylethanolamides in human seminal plasma by ultra performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography B,* 878**,** 3231-3237.

AMOAKO, A. A., MARCZYLO, T. H., MARCZYLO, E. L., ELSON, J., WILLETS, J. M., TAYLOR, A. H. & KONJE, J. C. 2013b. Anandamide modulates human sperm motility: implications for men with asthenozoospermia and oligoasthenoteratozoospermia. *Human Reproduction,* 28**,** 2058-2066.

ANDERSON, J. E., FARR, S. L., JAMIESON, D. J., WARNER, L. & MACALUSO, M. 2009. Infertility services reported by men in the United States: national survey data. *Fertility and Sterility,* 91**,** 2466-2470.

APPELL, R. A. & EVANS, P. R. 1977. The effect of temperature on sperm motility and viability. *Fertility and sterility,* 28**,** 1329-32.

ASHIZAWA, K., HASHIMOTO, K., HIGASHIO, M. & TSUZUKI, Y. 1997. The Addition of Mitogen-Activated Protein Kinase and p34cdc2Kinase Substrate Peptides Inhibits the Flagellar Motility of Demembranated Fowl Spermatozoa. *Biochemical and Biophysical Research Communications,* 240**,** 116-121.

ASHTON, J. C. & GLASS, M. 2007. The Cannabinoid CB2 Receptor as a Target for Inflammation-Dependent Neurodegeneration. *Curr Neuropharmacol,* 5**,** 73-80.

AURELL, C. & MEIZEL, W. S. 1993. Evidence Suggesting Involvement of a Unique Human Sperm Steroid Receptor/Cl- Channel Complex in the Progesterone-Initiated Acrosome Reaction. *Developmental Biology,* 159**,** 679-690.

AUSTIN, C. R. 1951. Observations on the penetration of the sperm in the mammalian egg. *Australian journal of scientific research. Ser. B: Biological sciences,* 4**,** 581-96.

AVIDAN, N., TAMARY, H., DGANY, O., CATTAN, D., PARIENTE, A., THULLIEZ, M., BOROT, N., MOATI, L., BARTHELME, A., SHALMON, L., KRASNOV, T., ASHER, E. B., OLENDER, T., KHEN, M., YANIV, I., ZAIZOV, R., SHALEV, H., DELAUNAY, J., FELLOUS, M., LANCET, D. & BECKMANN, J. S. 2003. CATSPER2, a human autosomal nonsyndromic male infertility gene. *European Journal of Human Genetics,* 11**,** 497-502.

BAJPAI, M. & DONCEL, G. F. 2003. Involvement of tyrosine kinase and cAMP-dependent kinase cross-talk in the regulation of human sperm motility. *Reproduction,* 126**,** 183-195.

BALDI, E., CASANO, R., FALSETTI, C., KRAUSZ, C., MAGGI, M. & FORTI, G. 1991. INTRACELLULAR CALCIUM ACCUMULATION AND RESPONSIVENESS TO PROGESTERONE IN CAPACITATING HUMAN SPERMATOZOA. *Journal of Andrology,* 12**,** 323-330.

BALDI, E., LUCONI, M., BONACCORSI, L. & FORTI, G. 1998. Nongenomic effects of progesterone on spermatozoa: Mechanisms of signal transduction and clinical implications. *Frontiers in Bioscience,* 3.

BARRATT, C. L. R. 2011. The mystery is solved-CatSper is the principal calcium channel activated by progesterone in human spermatozoa. *Asian Journal of Andrology,* 13**,** 351-352.

BARRATT, C. L. R., MANSELL, S., BEATON, C., TARDIF, S. & OXENHAM, S. K. 2011. Diagnostic tools in male infertility-the question of sperm dysfunction. *Asian Journal of Andrology,* 13**,** 53-58.

BARRATT, C. L. R., OSBORN, J. C., HARRISON, P. E., MONKS, N., DUNPHY, B. C., LENTON, E. A. & COOKE, I. D. 1989. THE HYPO-OSMOTIC SWELLING TEST AND THE SPERM MUCUS

PENETRATION TEST IN DETERMINING FERTILIZATION OF THE HUMAN OOCYTE. *Human Reproduction,* 4**,** 430-434.

BARRATT, C. L. R. & PUBLICOVER, S. J. 2012. Sperm are promiscuous and CatSper is to blame. *Embo Journal,* 31.

BATTISTONE, M. A., DA ROS, V. G., SALICIONI, A. M., NAVARRETE, F. A., KRAPF, D., VISCONTI, P. E. & CUASNICÚ, P. S. 2013. Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. *Molecular human reproduction***,** gat033--gat033-.

BAVISTER, B. D. 2002. Early history of in vitro fertilization. *Reproduction,* 124**,** 181-196.

BEAVO, J. A. 1995. CYCLIC-NUCLEOTIDE PHOSPHODIESTERASES - FUNCTIONAL IMPLICATIONS OF MULTIPLE ISOFORMS. *Physiological Reviews,* 75**,** 725-748.

BERGER, T., MARRS, R. P. & MOYER, D. L. 1985. COMPARISON OF TECHNIQUES FOR SELECTION OF MOTILE SPERMATOZOA. *Fertility and Sterility,* 43**,** 268-273.

BERWICK, D. C. & HARVEY, K. 2011. LRRK2 signaling pathways: the key to unlocking neurodegeneration? *Trends in Cell Biology,* 21**,** 257-265.

BIOSCIENCES, T. 2013a. *EO 1428 Supplier | CAS 321351-00-2 | Tocris Bioscience | EO1428* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5402 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013b. *GP 1a Supplier | Tocris Bioscience | GP1a* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5469 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013c. *JX 401 Supplier | CAS 349087-34-9 | Tocris Bioscience | JX401* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=5188 [Accessed 12th August 2013].

BIOSCIENCES, T. 2013d. *Leelamine Supplier | CAS 1446-61-3 | Tocris Bioscience | Dehydroabietylamine | Lylamine hydrochloride* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=109462 [Accessed 5th March 2013].

BIOSCIENCES, T. 2013e. *PHA 665752 Supplier | CAS 477575-56-7 | Tocris Bioscience | PHA665752* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=88278 [Accessed 13th August 2013].

BIOSCIENCES, T. 2013f. *Trequinsin Supplier | CAS 78416-81-6 | Tocris Bioscience | HL-725* [Online]. Available: http://www.tocris.com/dispprod.php?ItemId=142512 [Accessed 13th August 2013].

BJORNDAHL, L., MORTIMER, D., BARRATT, C. L. R., CASTILLA, J. A., MENKVELD, R., KVIST, U., ALVAREZ, J. G. & HAUGEN, T. B. 2010. *A Practical Guide to Basic Laboratory Andrology,* United Kingdom, Cambridge University Press.

BOATMAN, E. & ROBBINS, R. S. 1991a. Bicarbonate : Carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biology of reproduction,* 44**,** 806-813.

BOATMAN, E. & ROBBINS, R. S. 1991b. Bicarbonate : Regulation and of Sperm Hyperactivated Acrosome. *Biology of reproduction,* 44**,** 806-813.

BOIVIN, J., BUNTING, L., COLLINS, J. A. & NYGREN, K. G. 2007. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Human Reproduction,* 22**,** 1506-1512.

BOTTARO, D. P., RUBIN, J. S., FALETTO, D. L., CHAN, A. M. L., KMIECIK, T. E., VANDEWOUDE, G. F. & AARONSON, S. A. 1991. IDENTIFICATION OF THE HEPATOCYTE GROWTH-FACTOR RECEPTOR AS THE C-MET PROTOONCOGENE PRODUCT. *Science,* 251**,** 802-804.

BRAYTON, C. F. 1986. Dimethyl sulfoxide (DMSO): a review. *The Cornell veterinarian,* 76**,** 61-90.

BREITBART, H. 2002. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Molecular and Cellular Endocrinology,* 187**,** 139-144.

BRENKER, C., GOODWIN, N., WEYAND, I., KASHIKAR, N. D., NARUSE, M., KRAHLING, M., MULLER, A., KAUPP, U. B. & STRUNKER, T. 2012. The CatSper channel: a polymodal chemosensor in human sperm. *Embo Journal,* 31**,** 1654-1665.

BREWIS, I. A., MORTON, I. E., MOHAMMAD, S. N., BROWES, C. E. & MOORE, H. D. M. 2000. Measurement of intracellular calcium concentration and plasma membrane potential in human spermatozoa using flow cytometry. *Journal of Andrology,* 21**,** 238-249.

BRONSON, R. A., PERESLENI, T. & GOLIGHTLY, M. 1999. Progesterone promotes the acrosome reaction in capacitated human spermatozoa as judged by flow cytometry and CD46 staining. *Molecular Human Reproduction,* 5**,** 507-512.

BURKMAN, L. J. 1991. DISCRIMINATION BETWEEN NONHYPERACTIVATED AND CLASSICAL HYPERACTIVATED MOTILITY PATTERNS IN HUMAN SPERMATOZOA USING COMPUTERIZED ANALYSIS. *Fertility and Sterility,* 55**,** 363-371.

BUZADZIC, B., VUCETIC, M., JANKOVIC, A., STANCIC, A., KORAC, A., KORAC, B. & OTASEVIC, V. 2014. New insights into male (in)fertility: The importance of NO. *British Journal of Pharmacology***,** n/a-n/a.

CALANDRA, B., PORTIER, M., KERNÉIS, A., DELPECH, M., CARILLON, C., LE FUR, G., FERRARA, P. & SHIRE, D. 1999. Dual intracellular signaling pathways mediated by the human cannabinoid CB1 receptor. *European Journal of Pharmacology,* 374**,** 445-455.

CALOGERO, A. E., BURRELLO, N., BARONE, N., PALERMO, I., GRASSO, U. & D'AGATA, R. 2000. Effects of progesterone on sperm function: mechanisms of action. *Human Reproduction,* 15**,** 28-45.

CALOGERO, A. E., HALL, J., FISHEL, S., GREEN, S., HUNTER, A. & D'AGATA, R. 1996. Effects of γ-aminobutyric acid on human sperm motility and hyperactivation. *Molecular Human Reproduction,* 2**,** 733-738.

CAMPANA, A., SAKKAS, D., STALBERG, A., BIANCHI, P. G., COMTE, I., PACHE, T. & WALKER, D. 1996. Intrauterine insemination: evaluation of the results according to the woman's age, sperm quality, total sperm count per insemination and life table analysis. *Human Reproduction,* 11**,** 732-736.

CARLSON, A. E., BURNETT, L. A., DEL CAMINO, D., QUILL, T. A., HILLE, B., CHONG, J. A., MORAN, M. M. & BABCOCK, D. F. 2009. Pharmacological Targeting of Native CatSper Channels Reveals a Required Role in Maintenance of Sperm Hyperactivation. *PLoS ONE,* 4**,** e6844.

CARRERA, A., MOOS, J., NING, X. P., GERTON, G. L., TESARIK, J., KOPF, G. S. & MOSS, S. B. 1996. Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: Identification of a kinase anchor proteins as major substrates for tyrosine phosphorylation. *Developmental Biology,* 180**,** 284-296.

CASTILLA, J. A., ALVAREZ, C., AGUILAR, J., GONZÁLEZ-VAREA, C., GONZALVO, M. C. & MARTÍNEZ, L. 2006. Influence of analytical and biological variation on the clinical interpretation of seminal parameters. *Human reproduction (Oxford, England),* 21**,** 847-51.

CATIZONE, A., RICCI, G., DEL BRAVO, J. & GALDIERI, M. 2006. Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. *Journal of Endocrinology,* 189**,** 137-146.

CATTERALL, W. A. 2000. STRUCTURE AND REGULATION OF VOLTAGE-GATED Ca2+ CHANNELS. *Annual Review of Cell and Developmental Biology,* 16**,** 521-555.

CATTERALL, W. A. & FEW, A. P. 2008. Calcium Channel Regulation and Presynaptic Plasticity. *Neuron,* 59**,** 882-901.

CHANG, M. C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature,* 168**,** 697-8.

CHAPEAU, C. & GAGNON, D. C. 1987. Nitrocellulose and Polyvinyl Coatings Prevent Sperm Adhesion to Glass Without Affecting the Motility of Intact and Demembranated Human Spermatozoa. *Journal of Andrology,* 8**,** 34-40.

CHEMES, H. E., BRUGO OLMEDO, S., CARRERE, C., OSES, R., CARIZZA, C., LEISNER, M. & BLAQUIER, J. 1998. Ultrastructural pathology of the sperm flagellum: association

between flagellar pathology and fertility prognosis in severely asthenozoospermic men. *Human Reproduction,* 13**,** 2521-2526.

CHEN, Y. Q., CANN, M. J., LITVIN, T. N., IOURGENKO, V., SINCLAIR, M. L., LEVIN, L. R. & BUCK, J. 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science,* 289**,** 625-628.

CHULAVATNATOL, M. & HAESUNGCHARERN, A. 1977. Stabilization of adenylate energy charge and its relation to human sperm motility. *Journal of Biological Chemistry,* 252**,** 8088-8091.

COMHAIRE, F., MILINGOS, S., LIAPI, A., GORDTS, S., CAMPO, R., DEPYPERE, H., DHONT, M. & SCHOONJANS, F. 1995. The effective cumulative pregnancy rate of different modes of treatment of male infertility. *Andrologia,* 27**,** 217-221.

COOPER, T. G., NOONAN, E., VON ECKARDSTEIN, S., AUGER, J., BAKER, H. W. G., BEHRE, H. M., HAUGEN, T. B., KRUGER, T., WANG, C., MBIZVO, M. T. & VOGELSONG, K. M. 2010. World Health Organization reference values for human semen characteristics. *Human Reproduction Update,* 16**,** 231-245.

COSKRAN, T. M., MORTON, D., MENNITI, F. S., ADAMOWICZ, W. O., KLEIMAN, R. J., RYAN, A. M., STRICK, C. A., SCHMIDT, C. J. & STEPHENSON, D. T. 2006. Immunohistochemical Localization of Phosphodiesterase 10A in Multiple Mammalian Species. *Journal of Histochemistry & Cytochemistry,* 54**,** 1205-1213.

COSTELLO, S., MICHELANGELI, F., NASH, K., LEFIEVRE, L., MORRIS, J., MACHADO-OLIVEIRA, G., BARRATT, C., KIRKMAN-BROWN, J. & PUBLICOVER, S. 2009. Ca2+-stores in sperm: their identities and functions. *Reproduction,* 138**,** 425-437.

CRAVATT, B. F. & LICHTMAN, A. H. 2002. The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chemistry and Physics of Lipids,* 121**,** 135-148.

CURI, S. M., ARIAGNO, J. I., CHENLO, P. H., MENDELUK, G. R., PUGLIESE, M. N., SARDI SEGOVIA, L. M., REPETTO, H. E. H. & BLANCO, A. M. 2003. Asthenozoospermia: analysis of a large population. *Archives of andrology,* 49**,** 343-9.

DAI, S., HALL, D. D. & HELL, J. W. 2009. Supramolecular Assemblies and Localized Regulation of Voltage-Gated Ion Channels. *Physiological Reviews,* 89**,** 411-452.

DARSZON, A., ACEVEDO, J. J., GALINDO, B. E., HERNÁNDEZ-GONZÁLEZ, E. O., NISHIGAKI, T., TREVIÑO, C. L., WOOD, C. & BELTRÁN, C. 2006. Sperm channel diversity and functional multiplicity. *Reproduction,* 131**,** 977-988.

DARSZON, A., GUERRERO, A., GALINDO, B. E., NISHIGAKI, T. & WOOD, C. D. 2008. Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility. *International Journal of Developmental Biology,* 52**,** 595-606.

DARSZON, A., NISHIGAKI, T., BELTRAN, C. & TREVIÑO, C. L. 2011. Calcium channels in the development, maturation, and function of spermatozoa. *Physiological reviews,* 91**,** 1305-55.

DASCAL, N. 2001. Ion-channel regulated by G proteins. *TRENDS in Edocrinology & Metabolism,* 12**,** 391-398.

DAVID, G., SERRES, C. & JOUANNET, P. 1981. Kinematics of human spermatozoa. *Gamete Research,* 4**,** 83-95.

DE JONGE, C. 2005. Biological basis for human capacitation. *Human Reproduction Update,* 11**,** 205-214.

DE LAMIRANDE, E. & GAGNON, C. 2002. The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. *Molecular human reproduction,* 8**,** 124-35.

DE LAMIRANDE, E. & O'FLAHERTY, C. 2008. Sperm activation: Role of reactive oxygen species and kinases. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* 1784**,** 106-115.

DELAMIRANDE, E., LECLERC, P. & GAGNON, C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular Human Reproduction,* 3**,** 175-194.

DEMOTT, R. P. & SUAREZ, S. S. 1992. Hyperactivated sperm progress in the mouse oviduct. *Biology of Reproduction,* 46**,** 779-785.

DEPUYDT, C. E., DE POTTER, C. R., ZALATA, A., BAEKELANDT, E., BOSMANS, E. & COMHAIRE, F. H. 1998. Levels of Hepatocyte Growth Factor/Scatter Factor (HGF/SF) in Seminal Plasma of Patients With Andrological Diseases. *Journal of Andrology,* 19**,** 175-182.

DEPUYDT, C. E., ZALATA, A., DE POTTER, C. R., VAN EMMELO, J. & COMHAIRE, F. H. 1996. Genetic regulation of gametogensis: The receptor encoded by the human C-MET oncogene is expressed in testicular tissue and on human spermatozoa. *Molecular Human Reproduction,* 2**,** 2-8.

DEPUYDT, C. E., ZALATA, A., FALMAGNE, J. B., BOSMANS, E. & COMHAIRE, F. H. 1997. Purification and characterization of hepatocyte growth factor (HGF) from human seminal plasma. *International Journal of Andrology,* 20**,** 306-314.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. 1992. ISOLATION AND STRUCTURE OF A BRAIN CONSTITUENT THAT BINDS TO THE CANNABINOID RECEPTOR. *Science,* 258**,** 1946-1949.

DONNELLY, E. T. 2000. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. *Human Reproduction,* 15**,** 1552-1561.

DONNELLY, E. T., LEWIS, S. E., MCNALLY, J. A. & THOMPSON, W. 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. *Fertility and sterility,* 70**,** 305-14.

DOUGLAS-HAMILTON, D. H., SMITH, N. G., KUSTER, C. E., VERMEIDEN, J. P. W. & ALTHOUSE, G. C. 2005a. Capillary-Loaded Particle Fluid Dynamics: Effect on Estimation of Sperm Concentration. *Journal of Andrology,* 26**,** 115-122.

DOUGLAS-HAMILTON, D. H., SMITH, N. G., KUSTER, C. E., VERMEIDEN, J. P. W. & ALTHOUSE, G. C. 2005b. Particle Distribution in Low-Volume Capillary-Loaded Chambers. *Journal of Andrology,* 26**,** 107-114.

EGGERT-KRUSE, W., GERHARD, I., TILGEN, W. & RUNNEBAUM, B. 1989. CLINICAL-SIGNIFICANCE OF CROSSED INVITRO SPERM-CERVICAL MUCUS PENETRATION TEST IN INFERTILITY INVESTIGATION. *Fertility and Sterility,* 52**,** 1032-1040.

ESCOFFIER, J., BOISSEAU, S., SERRES, C., CHEN, C.-C., KIM, D., STAMBOULIAN, S., SHIN, H.-S., CAMPBELL, K. P., DE WAARD, M. & ARNOULT, C. 2007. Expression, localization and functions in acrosome reaction and sperm motility of CaV3.1 and CaV3.2 channels in sperm cells: An evaluation from CaV3.1 and CaV3.2 deficient mice. *Journal of Cellular Physiology,* 212**,** 753-763.

ESFANDIARI, N., SALEH, R. A., BLAUT, A. P., SHARMA, R. K., NELSON, D. R., THOMAS, A. J., FALCONE, T. & AGARWAL, A. 2002. Effects of temperature on sperm motion characteristics and reactive oxygen species. *International journal of fertility and women's medicine,* 47**,** 227-33.

ESHRE, E. S. O. H. R. A. E. 2012. **The world's number of IVF and ICSI babies has now reached a calculated total of 5 million**.

ESPOSITO, G., JAISWAL, B. S., XIE, F., KRAJNC-FRANKEN, M. A. M., ROBBEN, T., STRIK, A. M., KUIL, C., PHILIPSEN, R. L. A., VAN DUIN, M., CONTI, M. & GOSSEN, J. A. 2004. Mice deficient for soluble adenylyl cyclase are infertile because of a severe sperm-motility defect. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 2993-2998.

FAWCETT, L., BAXENDALE, R., STACEY, P., MCGROUTHER, C., HARROW, I., SODERLING, S., HETMAN, J., BEAVO, J. A. & PHILLIPS, S. C. 2000. Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proceedings of the National Academy of Sciences,* 97**,** 3702-3707.

FISCH, J. D., BEHR, B. & CONTI, M. 1998. Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. *Human Reproduction,* 13**,** 1248-1254.

FISHER, D. A., SMITH, J. F., PILLAR, J. S., ST. DENIS, S. H. & CHENG, J. B. 1998. Isolation and Characterization of PDE8A, a Novel Human cAMP-Specific Phosphodiesterase. *Biochemical and Biophysical Research Communications,* 246**,** 570-577.

FORD, W. C. L., REES, J. M., MCLAUGHLIN, E. A., LING, L. & HULL, M. G. R. 1994. Pentoxifylline acts synergistically with A23187 to increase the penetration of zona-free hamster oocytes by cryopreserved human spermatozoa. *International Journal of Andrology,* 17**,** 199-204.

FORSTER, M. S., SMITH, W. D., LEE, W. I., BERGER, R. E., KARP, L. E. & STENCHEVER, M. A. 1983. SELECTION OF HUMAN-SPERMATOZOA ACCORDING TO THEIR RELATIVE MOTILITY AND THEIR INTERACTION WITH ZONA-FREE HAMSTER EGGS. *Fertility and Sterility,* 40**,** 655-660.

FRANCAVILLA, F., BATTISTA, N., BARBONETTI, A., VASSALLO, M. R. C., RAPINO, C., ANTONANGELO, C., PASQUARIELLO, N., CATANZARO, G., BARBONI, B. & MACCARRONE, M. 2009. Characterization of the Endocannabinoid System in Human Spermatozoa and Involvement of Transient Receptor Potential Vanilloid 1 Receptor in Their Fertilizing Ability. *Endocrinology,* 150**,** 4692-4700.

FRASER, C. G. 2001. *Biological variation: from principles to practice*, Amer. Assoc. for Clinical Chemistry.

FRASER, L., BARRATT, C. L., CANALE, D., COOPER, T., DEJONGE, C., IRVINE, S., MORTIMER, D., OEHNINGER, S. & TESARIK, J. 1997. Consensus workshop on advanced diagnostic andrology techniques. ESHRE Andrology Special Interest Group. *Human Reproduction,* 12**,** 873-873.

FRASER, L. & GROUP, S. I. 1998. Guidelines on the application of CASA technology in the analysis of spermatozoa. 13**,** 142-145.

FRASER, L. R. 1977. Motility patterns in mouse spermatozoa before and after capacitation. *J Exp Zool,* 202**,** 439-44.

FRASER, L. R. 1998. Sperm capacitation and the acrosome reaction. *Human Reproduction,* 13.

GADELLA, B. M. & VISCONTI, P. E. 2006. Regulation of capacitation. *In:* BARRATT, C. L. R. & DE JONGE, C. J. (eds.). Cambridge: Cambridge University Press.

GARNIER-LHOMME, M., BYRNE, R. D., HOBDAY, T. M. C., GSCHMEISSNER, S., WOSCHOLSKI, R., POCCIA, D. L., DUFOURC, E. J. & LARIJANI, B. 2009. Nuclear Envelope Remnants: Fluid Membranes Enriched in STEROLS and Polyphosphoinositides. *Plos One,* 4.

GARRETT, C., LIU, D. Y., CLARKE, G. N., RUSHFORD, D. D. & BAKER, H. W. G. 2003. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. *Human Reproduction,* 18**,** 1643-1649.

GHALENO, L. R., VALOJERDI, M. R., CHEHRAZI, M. & SHARBATOGHLI, M. 2013. Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. *Archives of Gynecology and Obstetrics***,** 1-8.

GLASS, M. & FELDER, C. C. 1997. Concurrent Stimulation of Cannabinoid CB1 and Dopamine D2 Receptors Augments cAMP Accumulation in Striatal Neurons: Evidence for a Gs Linkage to the CB1 Receptor. *The Journal of Neuroscience,* 17**,** 5327-5333.

GOODWIN, L. O., KARABINUS, D. S., PERGOLIZZI, R. G. & BENOFF, S. 2000. L-type voltage-dependent calcium channel α-1C subunit mRNA is present in ejaculated human spermatozoa. *Molecular Human Reproduction,* 6**,** 127-136.

GOVERDE, A. J., MCDONNELL, J., VERMEIDEN, J. P. W., SCHATS, R., RUTTEN, F. F. H. & SCHOEMAKER, J. 2000. Intrauterine insemination or in-vitro fertilisation in idiopathic subfertility and male subfertility: a randomised trial and cost-effectiveness analysis. *The Lancet,* 355**,** 13-18.

GUERIN, J. F., MATHIEU, C., LORNAGE, J., PINATEL, M. C. & BOULIEU, D. 1989. IMPROVEMENT OF SURVIVAL AND FERTILIZING-CAPACITY OF HUMAN-SPERMATOZOA IN AN IVF

PROGRAM BY SELECTION ON DISCONTINUOUS PERCOLL GRADIENTS. *Human Reproduction,* 4**,** 798-804.

GUTKIND, J. S. 1998. The Pathways Connecting G Protein-coupled Receptors to the Nucleus through Divergent Mitogen-activated Protein Kinase Cascades. *Journal of Biological Chemistry,* 273**,** 1839-1842.

GUZICK, D. S., OVERSTREET, J. W., FACTOR -L ITVAK, P. A. M., BRAZIL, C. K., NAKAJIMA, S. T., COUTIFARIS, C., CARSON, S. A., CISNEROS, P., STEINKAMPF, M. P., HILL, J. A., XU, D. & VOGEL, D. L. 2001. Sperm morphology, Motility, and Concentration in Fertile and Infertile Men. *The New England journal of Medicine,* 345**,** 1388-1393.

HAMAMAH, S., MAGNOUX, E., ROYERE, D., BARTHELEMY, C., DACHEUX, J. L. & GATTI, J. L. 1996. Internal pH of human spermatozoa: effect of ions, human follicular fluid and progesterone. *Molecular human reproduction,* 2**,** 219-24.

HARGREAVE, T. B. & ELTON, R. A. 1983. Is conventional sperm analysis of any use? *British journal of urology,* 55**,** 774-9.

HARPER, C. V., BARRATT, C. L. R. & PUBLICOVER, S. J. 2004. Stimulation of Human Spermatozoa with Progesterone Gradients to Simulate Approach to the Oocyte: INDUCTION OF [Ca2+]i OSCILLATIONS AND CYCLICAL TRANSITIONS IN FLAGELLAR BEATING. *Journal of Biological Chemistry,* 279**,** 46315-46325.

HERNESS, E. A. & NAZ, R. K. 1999. Presence and Tyrosine Phosphorylation of c-met Receptor in Human Sperm. *Journal of Andrology,* 20**,** 640-647.

HESS, R. A. & RENATO DE FRANCA, L. 2008. Spermatogenesis and cycle of the seminiferous epithelium. *Advances in experimental medicine and biology,* 636**,** 1-15.

HEUCHEL, V., SCHWARTZ, D. & CZYGLIK, F. 1983. BETWEEN AND WITHIN SUBJECT CORRELATIONS AND VARIANCES FOR CERTAIN SEMEN CHARACTERISTICS IN FERTILE MEN. *Andrologia,* 15**,** 171-176.

HILDEBRAND, M. S., AVENARIUS, M. R., FELLOUS, M., ZHANG, Y., MEYER, N. C., AUER, J., SERRES, C., KAHRIZI, K., NAJMABADI, H., BECKMANN, J. S. & SMITH, R. J. H. 2010. Genetic male infertility and mutation of CATSPER ion channels. *European Journal of Human Genetics,* 18.

HO, H. 2001. Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction,* 122**,** 519-526.

HO, H.-C. & SUAREZ, S. S. 2001. An Inositol 1,4,5-Trisphosphate Receptor-Gated Intracellular Ca2+ Store Is Involved in Regulating Sperm Hyperactivated Motility. *Biology of Reproduction,* 65**,** 1606-1615.

HOOGEWIJS, M. K., DE VLIEGHER, S. P., GOVAERE, J. L., DE SCHAUWER, C., DE KRUIF, A. & VAN SOOM, A. 2012. Influence of counting chamber type on CASA outcomes of equine semen analysis. *Equine veterinary journal,* 44**,** 542-9.

HOWLETT, A. C. 2005. Cannabinoids. *In:* PERTWEE, R. (ed.) *Handbook of Experimental Pharmacology.* Heidelberg, Germany: Springer-Verlag.

HOWLETT, A. C., BARTH, F., BONNER, T. I., CABRAL, G., CASELLAS, P., DEVANE, W. A., FELDER, C. C., HERKENHAM, M., MACKIE, K., MARTIN, B. R., MECHOULAM, R. & PERTWEE, R. G. 2002. International Union of Pharmacology. XXVII. Classification of Cannabinoid Receptors. *Pharmacological Reviews,* 54**,** 161-202.

HULL, M. G. R., GLAZENER, C. M. A., KELLY, N. J., CONWAY, D. I., FOSTER, P. A., HINTON, R. A., COULSON, C., LAMBERT, P. A., WATT, E. M. & DESAI, K. M. 1985. Population study of causes, treatment, and outcome of infertility. *British Medical Journal,* 291**,** 1693-1697.

IKAWA, M., INOUE, N., BENHAM, A. M. & OKABE, M. 2010. Fertilization: a sperm's journey to and interaction with the oocyte. *Journal of Clinical Investigation,* 120**,** 984-994.

IMAI, H., SUZUKI, K., ISHIZAKA, K., ICHINOSE, S., OSHIMA, H., OKAYASU, I., EMOTO, K., UMEDA, M. & NAKAGAWA, Y. 2001. Failure of the Expression of Phospholipid Hydroperoxide Glutathione Peroxidase in the Spermatozoa of Human Infertile Males. *Biology of Reproduction,* 64**,** 674-683.

IRVINE, D. S. 1998. Epidemiology and aetiology of male infertility. *Human Reproduction,* 13**,** 33-44.

IVIC, A. 2002. Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Human Reproduction,* 17**,** 143-149.

JAISWAL, B. S. & MAJUMDER, G. C. 1996. Cyclic AMP phosphodiesterase: A regulator of forward motility initiation during epididymal sperm maturation. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire,* 74**,** 669-674.

JEDRZEJCZAK, P., TASZAREK-HAUKE, G., HAUKE, J., PAWELCZYK, L. & DULEBA, A. J. 2008. Prediction of spontaneous conception based on semen parameters. *International Journal of Andrology,* 31**,** 499-507.

JEYENDRAN, R. S., VAN DER VEN, H. H., PEREZ-PELAEZ, M., CRABO, B. G. & ZANEVELD, L. J. D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility,* 70**,** 219-228.

JOHNSON, L. L., KATZ, D. F. & OVERSTREET, J. W. 1981. The movement characteristics of rabbit spermatozoa before and after activation. *Gamete Research,* 4**,** 275-282.

JØRGENSEN, N., ANDERSEN, A. G., EUSTACHE, F., IRVINE, D. S., SUOMINEN, J., PETERSEN, J. H., ANDERSEN, A. N., AUGER, J., CAWOOD, E. H., HORTE, A., JENSEN, T. K., JOUANNET, P., KEIDING, N., VIERULA, M., TOPPARI, J. & SKAKKEBAEK, N. E. 2001. Regional differences in semen quality in Europe. *Human reproduction (Oxford, England),* 16**,** 1012-9.

KARANDE, V. C., KORN, A., MORRIS, R., RAO, R., BALIN, M., RINEHART, J., DOHN, K. & GLEICHER, N. 1999. Prospective randomized trial comparing the outcome and cost of in vitro fertilization with that of a traditional treatment algorithm as first-line therapy for couples with infertility. *Fertility and Sterility,* 71**,** 468-475.

KATZ, D., OVERSTREET, J. & HANSON, F. 1980. A new quantitative test for sperm penetration into cervical mucus. *Fertility and Sterility,* 33**,** 179-186.

KAY, V. J., COUTTS, J. R. T. & ROBERTSON, L. 1993. PENTOXIFYLLINE STIMULATES HYPERACTIVATION IN HUMAN SPERMATOZOA. *Human Reproduction,* 8**,** 727-731.

KEEL, B. A. 2006. Within- and between-subject variation in semen parameters in infertile men and normal semen donors. *Fertility and Sterility,* 85**,** 128-134.

KIRICHOK, Y. & LISHKO, P. V. 2011. Rediscovering sperm ion channels with the patch-clamp technique. *Molecular Human Reproduction,* 17**,** 478-499.

KIRICHOK, Y., NAVARRO, B. & CLAPHAM, D. E. 2006. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. *Nature,* 439**,** 737-740.

KITAMURA, M., MATSUMIYA, K., NISHIMURA, K., YAMANAKA, M., MATSUMOTO, K. & OKUYAMA, A. 2000. Effect of hepatocyte growth factor on sperm motility. *American Journal of Reproductive Immunology,* 44**,** 193-196.

KRAEMER, M., FILLION, C., MARTIN-PONT, B. & AUGER, J. 1998. Factors influencing human sperm kinematic measurements by the Celltrak computer-assisted sperm analysis system. *Human reproduction (Oxford, England),* 13**,** 611-9.

KRASZNAI, Z., KRASZNAI, Z. T., MORISAWA, M., BAZSÁNÉ, Z. K., HERNÁDI, Z., FAZEKAS, Z., TRÓN, L., GODA, K. & MÁRIÁN, T. 2006. Role of the Na+/Ca2+ exchanger in calcium homeostasis and human sperm motility regulation. *Cell motility and the cytoskeleton,* 63**,** 66-76.

KRAUSZ, C., BONACCORSI, L., MAGGIO, P., LUCONI, M., CRISCUOLI, L., FUZZI, B., PELLEGRINI, S., FORTI, G. & BALDI, E. 1996. Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Human reproduction (Oxford, England),* 11**,** 1661-7.

KREMER, J. 1965. A SIMPLE SPERM PENETRATION TEST. *International Journal of Fertility,* 10**,** 209-&.

LAIL-TRECKER, M., GULATI, R. & PELUSO, J. J. 1998. A role for hepatocyte growth factor scatter factor in regulating normal and neoplastic cells of reproductive tissues. *Journal of the Society for Gynecologic Investigation,* 5**,** 114-121.

LAN, N., BOLGER, M. & GEE, K. 1991. Identification and characterization of a pregnane steroid recognition site that is functionally coupled to an expressed GABAA receptor. *Neurochemical Research,* 16**,** 347-356.

LARSEN, L., SCHEIKE, T., JENSEN, T. K. & BONDE, J. P. 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. *Human …,* 15**,** 1562-1567.

LE LANNOU, D., GRIVEAU, J. F., LE PICHON, J. P. & QUERO, J. C. 1992. Effects of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. *Human reproduction (Oxford, England),* 7**,** 1417-21.

LEE, M. A. & STOREY, B. T. 1986. BICARBONATE IS ESSENTIAL FOR FERTILIZATION OF MOUSE EGGS - MOUSE SPERM REQUIRE IT TO UNDERGO THE ACROSOME REACTION. *Biology of Reproduction,* 34**,** 349-356.

LEFIEVRE, L., BEDU-ADDO, K., CONNER, S. J., MACHADO-OLIVEIRA, G. S. M., CHEN, Y., KIRKMAN-BROWN, J. C., AFNAN, M. A., PUBLICOVER, S. J., FORD, W. C. L. & BARRATT, C. L. R. 2007. Counting sperm does not add up any more: time for a new equation? *Reproduction,* 133**,** 675-684.

LEFIEVRE, L., DE LAMIRANDE, E. & GAGNON, C. 2002. Presence of cyclic nucleotide phosphodiesterases PDE1, existing as a stable complex with calmodulin, and PDE3A in human spermatozoa. *Biology of Reproduction,* 67**,** 423-430.

LEFIEVRE, L., DE LAMIRANDE, E. V. E. & GAGNON, C. 2000. The Cyclic GMP-Specific Phosphodiesterase Inhibitor, Sildenafil, Stimulates Human Sperm Motility and Capacitation but Not Acrosome Reaction. *Journal of Andrology,* 21**,** 929-937.

LEFIEVRE, L., NASH, K., MANSELL, S., COSTELLO, S., PUNT, E., CORREIA, J., MORRIS, J., KIRKMAN-BROWN, J., WILSON, S., BARRATT, C. & PUBLICOVER, S. 2012. 2-APB-potentiated channels amplify CatSper-induced Ca2+ signals in human sperm. *Biochemical Journal,* 448**,** 189-200.

LEJA 2005. The Segre Silberberg effect.

LEWIS, S. E. M. 2007. Is sperm evaluation useful in predicting human fertility? *Reproduction,* 134**,** 31-40.

LI, M. W. M., MRUK, D. D. & CHENG, C. Y. 2009. Mitogen-activated protein kinases in male reproductive function. *Trends in Molecular Medicine,* 15**,** 159-168.

LINDEMANN, C. B. & KANOUS, K. S. 1989. REGULATION OF MAMMALIAN SPERM MOTILITY. *Archives of Andrology,* 23**,** 1-22.

LISHKO, P. V., BOTCHKINA, I. L., FEDORENKO, A. & KIRICHOK, Y. 2010. Acid Extrusion from Human Spermatozoa Is Mediated by Flagellar Voltage-Gated Proton Channel. *Cell,* 140**,** 327-337.

LISHKO, P. V., BOTCHKINA, I. L. & KIRICHOK, Y. 2011. Progesterone activates the principal Ca2+ channel of human sperm. *Nature,* 471**,** 387-391.

LISHKO, P. V. & KIRICHOK, Y. 2010. The role of Hv1 and CatSper channels in sperm activation. *The Journal of Physiology,* 588**,** 4667-4672.

LISHKO, P. V., KIRICHOK, Y., REN, D., NAVARRO, B., CHUNG, J.-J. & CLAPHAM, D. E. 2012a. The Control of Male Fertility by Spermatozoan Ion Channels. *Annual Review of Physiology,* 74**,** 453-475.

LISHKO, P. V., KIRICHOK, Y., REN, D. J., NAVARRO, B., CHUNG, J. J. & CLAPHAM, D. E. 2012b. The Control of Male Fertility by Spermatozoan Ion Channels. *In:* JULIUS, D. & CLAPHAM, D. E. (eds.) *Annual Review of Physiology, Vol 74.* Palo Alto: Annual Reviews.

LIU, D. Y. & BAKER, H. W. G. 1988. THE PROPORTION OF HUMAN-SPERM WITH POOR MORPHOLOGY BUT NORMAL INTACT ACROSOMES DETECTED WITH PISUM-SATIVUM AGGLUTININ CORRELATES WITH FERTILIZATION INVITRO. *Fertility and Sterility,* 50**,** 288-293.

LIU, D. Y., LIU, M. L., CLARKE, G. N. & BAKER, H. W. G. 2007. Hyperactivation of capacitated human sperm correlates with the zona pellucida-induced acrosome reaction of zona pellucida-bound sperm. *Human Reproduction,* 22**,** 2632-2638.

LIÉVANO, A., SANTI, C. M., SERRANO, C. J., TREVIÑO, C. L., BELLVÉ, A. R., HERNÁNDEZ-CRUZ, A. & DARSZON, A. 1996. T-type Ca2+ channels and α1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. *FEBS Letters,* 388**,** 150-154.

LOPEZ-GONZALEZ, I., DE LA VEGA-BELTRAN, J. L., SANTI, C. M., FLORMAN, H. M., FELIX, R. & DARSZON, A. 2001. Calmodulin antagonists inhibit T-type Ca2+ currents in mouse spermatogenic cells and the zona pellucida-induced sperm acrosome reaction. *Developmental Biology,* 236**,** 210-219.

LU, Q. & AN, W. F. 2008. Impact of novel screening technologies on ion channel drug discovery. *Combinatorial Chemistry & High Throughput Screening,* 11**,** 185-194.

MACCARRONE, M. 2008. CB2 receptors in reproduction. *British Journal of Pharmacology,* 153**,** 189-198.

MACLEOD, J. & GOLD, R. Z. 1951a. The male factor in fertility and infertility. II. Spermatozoon counts in 1000 men of known fertility and in 1000 cases of infertile marriage. *The Journal of urology,* 66**,** 436-49.

MACLEOD, J. & GOLD, R. Z. 1951b. The Male Factor in Fertility and Infertility. III. An Analysis of Motile Activity in the Spermatozoa of 1000 Fertile Men and 1000 Men in Infertile Marriage. *Fertility and sterility,* 2**,** 187-204.

MAHI, C. A. & YANAGIMACHI, R. 1978. Capacitation, acrosome reaction, and egg penetration by canine spermatozoa in a simple defined medium. *Gamete Research,* 1**,** 101-109.

MAHMOUD, A. M., DEPOORTER, B., PIENS, N. & COMHAIRE, F. H. 1997. The performance of 10 different methods for the estimation of sperm concentration. *Fertility and sterility,* 68**,** 340-5.

MAJEWSKA, M. D., HARRISON, N. L., SCHWARTZ, R. D., BARKER, J. L. & PAUL, S. M. 1986. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science,* 232**,** 1004-1007.

MAKLER, A., MAKLER, E., ITZKOVITZ, J. & BRANDES, J. M. 1980. Factors affecting sperm motility. IV. Incubation of human semen with caffeine, kallikrein, and other metabolically active compounds. *Fertility and sterility,* 33**,** 624-30.

MALLIDIS, C., HOWARD, E. J. & BAKER, H. W. G. 1991. VARIATION OF SEMEN QUALITY IN NORMAL MEN. *International Journal of Andrology,* 14**,** 99-107.

MANNOWETZ, N., NAIDOO, N. M., CHOO, S.-A. S., SMITH, J. F., LISHKO, P. V. & ALDRICH, R. 2013. Slo1 is the principal potassium channel of human spermatozoa. *eLife,* 2.

MANSELL, S. A., PUBLICOVER, S. J., BARRATT, C. L. R. & WILSON, S. M. 2014. Patch clamp studies of human sperm under physiological ionic conditions reveal three functionally and pharmacologically distinct cation channels. *Molecular Human Reproduction*.

MARTINS DA SILVA, S. J., HOPE, A. G., GRAY, D. W. & BARRATT, C. L. R. 2012. Validation of a novel high throughput screening assay to assess calcium responses in human sperm. *Fertility and Sterility,* 98**,** S84-S85.

MARZO, V. D., PETROCELLIS, L. D. & BISOGNO, T. 2005. The Biosynthesis, Fate and Pharmacological Properties of Endocannabinoids. *In:* PERTWEE, R. (ed.) *Cannabinoids: Handbook of Experimental Pharmacology.* Germany: Springer- Verlag.

MARÍN-BRIGGILER, C. I., JHA, K. N., CHERTIHIN, O., BUFFONE, M. G., HERR, J. C., VAZQUEZ-LEVIN, M. H. & VISCONTI, P. E. 2005. Evidence of the presence of calcium/calmodulin-dependent protein kinase IV in human sperm and its involvement in motility regulation. *Journal of Cell Science,* 118**,** 2013-2022.

MATSON, P. & TARDIF, S. 2012. A preliminary search for alternatives to albumin as a medium supplement for the culture of human sperm. *Reproductive Biology,* 12**,** 329-331.

MAYR, L. M. & BOJANIC, D. 2009. Novel trends in high-throughput screening. *Current Opinion in Pharmacology,* 9**,** 580-588.

MCLESKEY, S. B., DOWDS, C., CARBALLADA, R., WHITE, R. R. & SALING, P. M. 1998. Molecules involved in mammalian sperm-egg interaction. *International Review of Cytology - a Survey of Cell Biology, Vol 177,* 177.

MEIZEL, S., TURNER, K. O. & NUCCITELLI, R. 1997. Progesterone Triggers a Wave of Increased Free Calcium during the Human Sperm Acrosome Reaction. *Developmental Biology,* 182**,** 67-75.

MIKI, K., QU, W., GOULDING, E. H., WILLIS, W. D., BUNCH, D. O., STRADER, L. F., PERREAULT, S. D., EDDY, E. M. & O'BRIEN, D. A. 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 16501-16506.

MILARDI, D., GRANDE, G., SACCHINI, D., ASTORRI, A. L., POMPA, G., GIAMPIETRO, A., DE MARINIS, L., PONTECORVI, A., SPAGNOLO, A. G. & MARANA, R. 2012. Male fertility and reduction in semen parameters: a single tertiary-care center experience. *International journal of endocrinology,* 2012**,** 649149-649149.

MILLER, J. L. 2006. Recent developments in focused library design: Targeting gene-families. *Current Topics in Medicinal Chemistry,* 6**,** 19-29.

MOK, N. Y. & BRENK, R. 2011. Mining the ChEMBL Database: An Efficient Chemoinformatics Workflow for Assembling an Ion Channel-Focused Screening Library. *Journal of Chemical Information and Modeling,* 51**,** 2449-2454.

MOOHAN, J. M. & LINDSAY, K. S. 1995. SPERMATOZOA SELECTED BY A DISCONTINUOUS PERCOLL DENSITY GRADIENT EXHIBIT BETTER MOTION CHARACTERISTICS, MORE HYPERACTIVATION, AND LONGER SURVIVAL THAN DIRECT SWIM-UP. *Fertility and Sterility,* 64**,** 160-165.

MORISAWA, M. 1994. CELL SIGNALING MECHANISMS FOR SPERM MOTILITY. *Zoological Science,* 11**,** 647-662.

MORTIMER, D. 2000a. Sperm Preparation Methods. *Journal of Andrology,* 21**,** 357-366.

MORTIMER, D., AITKEN, R. J., MORTIMER, S. T. & PACEY, A. A. 1995. Workshop report: clinical CASA--the quest for consensus. *Reproduction, Fertility and Development,* 7**,** 951-951.

MORTIMER, D., COURTOT, A. M., GIOVANGRANDI, Y., JEULIN, C. & DAVID, G. 1984. Human sperm motility after migration into, and incubation in, synthetic media. *Gamete Research,* 9**,** 131-144.

MORTIMER, D. & MORTIMER, S. T. 2013a. Computer-Aided Sperm Analysis (CASA) of Sperm Motility and Hyperactivation. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, D. & MORTIMER, S. T. 2013b. Maunal Methods for Sperm Motility Assessment. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, D. & MORTIMER, S. T. 2013c. Spermatogenesis. *In:* CARRELL, D. T. & ASTON, K. I. (eds.). Totowa, NJ: Humana Press.

MORTIMER, S. T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. *Human Reproduction Update,* 3**,** 403-439.

MORTIMER, S. T. 2000b. CASA — Practical Aspects Andrology Lab Corner.

MORTIMER, S. T. & MORTIMER, D. 1990. Kinematics of Human Spermatozoa Incubated Under Capacitating Conditions. *Journal of Andrology,* 11**,** 195-203.

MORTIMER, S. T., SCHOEVAERT, D., SWAN, M. A. & MORTIMER, D. 1997. Quantitative observations of flagellar motility of capacitating human spermatozoa. *Human Reproduction,* 12**,** 1006-1012.

MORTIMER, S. T. & SWAN, M. A. 1995. Variable kinematics of capacitating human spermatozoa. *Human Reproduction,* 10**,** 3178-3182.

MORTIMER, S. T. & SWAN, M. A. 1999. Effect of image sampling frequency on established and smoothing-independent kinematic values of capacitating human spermatozoa. *Human Reproduction,* 14**,** 997-1004.

MORTIMER, S. T., SWAN, M. A. & MORTIMER, D. 1998. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Human Reproduction,* 13**,** 2139-2146.

MUKAI, C. & OKUNO, M. 2004. Glycolysis Plays a Major Role for Adenosine Triphosphate Supplementation in Mouse Sperm Flagellar Movement. *Biology of Reproduction,* 71**,** 540-547.

NALDINI, L., WEIDNER, K. M., VIGNA, E., GAUDINO, G., BARDELLI, A., PONZETTO, C., NARSIMHAN, R. P., HARTMANN, G., ZARNEGAR, R., MICHALOPOULOS, G. K., BIRCHMEIER, W. & COMOGLIO, P. M. 1991. SCATTER FACTOR AND HEPATOCYTE GROWTH-FACTOR ARE INDISTINGUISHABLE LIGANDS FOR THE MET RECEPTOR. *Embo Journal,* 10**,** 2867-2878.

NAVARRO, B., KIRICHOK, Y. & CLAPHAM, D. E. 2007. KSper, a pH-sensitive K+ current that controls sperm membrane potential. *Proceedings of the National Academy of Sciences,* 104**,** 7688-7692.

NAZ, R. K. 1996. Protein tyrosine phosphorylation and signal transduction during capacitation-acrosome reaction and zona pellucida binding in human sperm. *Archives of Andrology,* 37**,** 47-55.

NAZ, R. K., AHMAD, K. & KUMAR, R. 1991. ROLE OF MEMBRANE PHOSPHOTYROSINE PROTEINS IN HUMAN SPERMATOZOAL FUNCTION. *Journal of Cell Science,* 99**,** 157-165.

NAZ, R. K., JOSEPH, A., LEE, Y., AHMAD, K. & BHARGAVA, M. M. 1994. Expression of scatter factor/hepatocyte growth factor is regionally correlated with the initiation of sperm motility in murine male genital tract: Is scatter factor/hepatocyte growth factor involved in initiation of sperm motility? *Molecular Reproduction and Development,* 38**,** 431-439.

NAZ, R. K. & RAJESH, P. B. 2004. Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reproductive biology and endocrinology : RB&E,* 2**,** 75.

NICE, N. I. F. H. A. C. E. 2013. Fertility:assessment and treatment for people with fertility problems. 2nd edition ed. London: National Collaborating Centre for Women's and Children's Health.

O'FLAHERTY, C., DE LAMIRANDE, E. & GAGNON, C. 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radical Biology and Medicine,* 41**,** 528-540.

OACONNELL, M. 2003. Differences in mitochondrial and nuclear DNA status of high-density and low-density sperm fractions after density centrifugation preparation*1. *Fertility and Sterility,* 79**,** 754-762.

OKAMOTO, Y., WANG, J., MORISHITA, J. & UEDA, N. 2007. Biosynthetic pathways of the endocannabinoid anandamide. *Chemistry & Biodiversity,* 4**,** 1842-1857.

OLSON, S., SUAREZ, S. & FAUCI, L. 2010. A Model of CatSper Channel Mediated Calcium Dynamics in Mammalian Spermatozoa. *Bulletin of Mathematical Biology,* 72**,** 1925-1946.

OLSON, S. D., FAUCI, L. J. & SUAREZ, S. S. 2011. Mathematical modeling of calcium signaling during sperm hyperactivation. *Molecular Human Reproduction,* 17**,** 500-510.

OMBELET, W., BOSMANS, E., JANSSEN, M., COX, A., VLASSELAER, J., GYSELAERS, W., VANDEPUT, H., GIELEN, J., POLLET, H., MAES, M., STEENO, O. & KRUGER, T. 1997. Semen parameters in a fertile versus subfertile population: A need for change in the interpretation of semen testing. *Human Reproduction,* 12**,** 987-993.

PACEY, A. A., DAVIES, N., WARREN, M. A., BARRATT, C. L. R. & COOKE, L. D. 1995. Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. *Human Reproduction,* 10**,** 2603-2609.

PARK, J.-Y., AHN, H.-J., GU, J.-G., LEE, K.-H., KIM, J.-S., KANG, H.-W. & LEE, J.-H. 2003. Molecular identification of Ca(2+)channels in human sperm. *Exp Mol Med,* 35**,** 285-292.

PEARSON, G., ROBINSON, F., BEERS GIBSON, T., XU, B.-E., KARANDIKAR, M., BERMAN, K. & COBB, M. H. 2001. Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. *Endocrine Reviews,* 22**,** 153-183.

PERTWEE, R. G. 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology & Therapeutics,* 74**,** 129-180.

PERTWEE, R. G. 2005. Pharmacological Actions of Cannabinoids. *In:* PERTWEE, R. (ed.) *Cannabinoids.* Springer Berlin Heidelberg.

POLAND, M. L., MOGHISSI, K. S., GIBLIN, P. T., AGER, J. W. & OLSON, J. M. 1985. VARIATION OF SEMEN MEASURES WITHIN NORMAL MEN. *Fertility and Sterility,* 44**,** 396-400.

PORRAS, A., ZULUAGA, S., BLACK, E., VALLADARES, A., ALVAREZ, A. M., AMBROSINO, C., BENITO, M. & NEBREDA, A. R. 2004. p38α Mitogen-activated Protein Kinase Sensitizes Cells to Apoptosis Induced by Different Stimuli. *Molecular Biology of the Cell,* 15**,** 922-933.

PUBLICOVER, S., HARPER, C. V. & BARRATT, C. 2007. [Ca2+]i signalling in sperm-making the most of what you've got. *Nature Cell Biology,* 9**,** 235-42.

PUBLICOVER, S. J. & BARRATT, C. L. R. 2011. Sperm motility: things are moving in the lab! *Molecular Human Reproduction,* 17**,** 453-456.

QI, H., MORAN, M. M., NAVARRO, B., CHONG, J. A., KRAPIVINSKY, G., KRAPIVINSKY, L., KIRICHOK, Y., RAMSEY, I. S., QUILL, T. A. & CLAPHAM, D. E. 2007. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proceedings of the National Academy of Sciences,* 104**,** 1219-1223.

RAMMLER, D. H. & ZAFFARONI, A. 1967. BIOLOGICAL IMPLICATIONS OF DMSO BASED ON A REVIEW OF ITS CHEMICAL PROPERTIES. *Annals of the New York Academy of Sciences,* 141**,** 13-23.

RAUPRICH, O., BERNS, E. & VOLLMANN, J. 2010. Who should pay for assisted reproductive techniques? Answers from patients, professionals and the general public in Germany. *Human reproduction (Oxford, England),* 25**,** 1225-33.

REN, D. & XIA, J. 2010. Calcium Signaling Through CatSper Channels in Mammalian Fertilization. *Physiology,* 25**,** 165-175.

REN, D. J., NAVARRO, B., PEREZ, G., JACKSON, A. C., HSU, S. F., SHI, Q., TILLY, J. L. & CLAPHAM, D. E. 2001. A sperm ion channel required for sperm motility and male fertility. *Nature,* 413**,** 603-609.

RICHTER, W., DETTMER, D. & GLANDER, H. J. 1999. Detection of mRNA transcripts of cyclic nucleotide phosphodiesterase subtypes in ejaculated human spermatozoa. *Molecular Human Reproduction,* 5**,** 732-736.

RITTA, M. N., CALAMERA, J. C. & BAS, D. E. 1998. Occurrence of GABA and GABA receptors in human spermatozoa. *Molecular Human Reproduction,* 4**,** 769-773.

ROSSATO, M., ION POPA, F., FERIGO, M., CLARI, G. & FORESTA, C. 2005. Human Sperm Express Cannabinoid Receptor Cb1, the Activation of Which Inhibits Motility, Acrosome Reaction, and Mitochondrial Function. *Journal of Clinical Endocrinology & Metabolism,* 90**,** 984-991.

ROSSI, P., PEZZOTTI, R., CONTI, M. & GEREMIA, R. 1985. Cyclic nucleotide phosphodiesterases in somatic and germ cells of mouse seminiferous tubules. *Journal of Reproduction and Fertility,* 74**,** 317-327.

ROWE, P. C., FRANK. HARGREAVE, TIMOTHY. MELLOWS, HEATHER. 1993. *WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple,* Cambridge, Cambridge University Press.

SAAD, A. & GUERIN, J. F. 1992. Movement characteristics of human spermatozoa collected from different layers of a discontinuous Percoll gradient. *Andrologia,* 24**,** 149-153.

SAGARE-PATIL, V., GALVANKAR, M., SATIYA, M., BHANDARI, B., GUPTA, S. K. & MODI, D. 2012. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *International Journal of Andrology,* 35**,** 633-644.

SALICIONI, A. M., PLATT, M. D., WERTHEIMER, E. V., ARCELAY, E., ALLAIRE, A., SOSNIK, J. & VISCONTI, P. E. 2007. Signalling pathways involved in sperm capacitation. *Society of Reproduction and Fertility supplement,* 65**,** 245-59.

SANTI, C. M., MARTÍNEZ-LÓPEZ, P., DE LA VEGA-BELTRÁN, J. L., BUTLER, A., ALISIO, A., DARSZON, A. & SALKOFF, L. 2010. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. *FEBS Letters,* 584**,** 1041-1046.

SCHMIDT, L., MÜNSTER, K. & HELM, P. 1995. Infertility and the seeking of infertility treatment in a representative population. *British journal of obstetrics and gynaecology,* 102**,** 978-84.

SCHREIBER, M., WEI, A., YUAN, A., GAUT, J., SAITO, M. & SALKOFF, L. 1998. Slo3, a Novel pH-sensitive K+ Channel from Mammalian Spermatocytes. *Journal of Biological Chemistry,* 273**,** 3509-3516.

SCHREIBER, M., YUAN, A. & SALKOFF, L. 1999. Transplantable sites confer calcium sensitivity to BK channels. *Nature Neuroscience,* 2**,** 416-421.

SCHUEL, H., BURKMAN, L. J., LIPPES, J., CRICKARD, K., MAHONY, M. C., GIUFFRIDA, A., PICONE, R. P. & MAKRIYANNIS, A. 2002. Evidence that anandamide-signaling regulates human sperm functions required for fertilization. *Molecular Reproduction and Development,* 63**,** 376-387.

SERRANO, C. J., TREVIÑO, C. L., FELIX, R. & DARSZON, A. 1999. Voltage-dependent Ca2+ channel subunit expression and immunolocalization in mouse spermatogenic cells and sperm. *FEBS Letters,* 462**,** 171-176.

SERVIN-VENCES, M. R., TATSU, Y., ANDO, H., GUERRERO, A., YUMOTO, N., DARSZON, A. & NISHIGAKI, T. 2012. A caged progesterone analog alters intracellular Ca2+ and flagellar bending in human sperm. *Reproduction,* 144**,** 101-109.

SHI, Q. X. & ROLDAN, E. R. 1995. Evidence that a GABAA-like receptor is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. *Biology of Reproduction,* 52**,** 373-381.

SI, Y. & OKUNO, M. 1995. Activation of mammalian sperm motility by regulation of microtubule sliding via cyclic adenosine 5'-monophosphate-dependent phosphorylation. *Biology of Reproduction,* 53**,** 1081-1087.

SIGNORELLI, J., DIAZ, E. S. & MORALES, P. 2012. Kinases, phosphatases and proteases during sperm capacitation. *Cell and Tissue Research,* 349**,** 765-782.

SINGH, J. P., BABCOCK, D. F. & LARDY, H. A. 1983. Motility activation, respiratory stimulation, and alteration of Ca2+ transport in bovine sperm treated with amine local anesthetics and calcium transport antagonists. *Archives of Biochemistry and Biophysics,* 221**,** 291-303.

SODERLING, S. H. & BEAVO, J. A. 2000. Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Current Opinion in Cell Biology,* 12**,** 174-179.

STONE, B. A., VARGYAS, J. M., RINGLER, G. E., STEIN, A. L. & MARRS, R. P. 1999. Determinants of the outcome of intrauterine insemination: Analysis of outcomes of 9963 consecutive cycles. *American Journal of Obstetrics and Gynecology,* 180**,** 1522-1534.

STRAIN, A. J. 1993. HEPATOCYTE GROWTH-FACTOR - ANOTHER UBIQUITOUS CYTOKINE. *Journal of Endocrinology,* 137**,** 1-5.

STRUNKER, T., GOODWIN, N., BRENKER, C., KASHIKAR, N. D., WEYAND, I., SEIFERT, R. & KAUPP, U. B. 2011. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. *Nature,* 471**,** 382-386.

SUAREZ, S. S. 2008. Control of hyperactivation in sperm. *Human Reproduction Update,* 14**,** 647-657.

SUAREZ, S. S., KATZ, D. F., OWEN, D. H., ANDREW, J. B. & POWELL, R. L. 1991. Evidence for the function of hyperactivated motility in sperm. *Biology of Reproduction,* 44**,** 375-381.

SUAREZ, S. S., VAROSI, S. M. & DAI, X. 1993. Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *Proceedings of the National Academy of Sciences,* 90**,** 4660-4664.

SUELDO, C. E., OEHNINGER, S., SUBIAS, E., MAHONY, M., ALEXANDER, N. J., BURKMAN, L. J. & ACOSTA, A. A. 1993. Effect of progesterone on human zona pellucida sperm binding and oocyte penetrating capacity. *Fertility and sterility,* 60**,** 137-140.

SUGIURA, T., KODAKA, T., KONDO, S., NAKANE, S., KONDO, H., WAKU, K., ISHIMA, Y., WATANABE, K. & YAMAMOTO, I. 1997. Is the Cannabinoid CB1 Receptor a 2-Arachidonoylglycerol Receptor? Structural Requirements for Triggering a Ca2+ Transient in NG108–15 Cells. *Journal of Biochemistry,* 122**,** 890-895.

SUGIURA, T. & WAKU, K. 2000. 2-Arachidonoylglycerol and the cannabinoid receptors. *Chemistry and Physics of Lipids,* 108**,** 89-106.

SUKCHAROEN, N., KEITH, J., IRVINE, D. S. & AITKEN, R. J. 1995. PREDICTING THE FERTILIZING POTENTIAL OF HUMAN SPERM SUSPENSIONS IN-VITRO - IMPORTANCE OF SPERM MORPHOLOGY AND LEUKOCYTE CONTAMINATION. *Fertility and Sterility,* 63.

SUKCHAROEN, N., KEITH, J., IRVINE, D. S. & AITKEN, R. J. 1996. Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Human Reproduction,* 11**,** 1030-1034.

TARDIF, S., MADAMIDOLA, O. A., BROWN, S. G., FRAME, L., LEFIÈVRE, L., WYATT, P. G., BARRATT, C. L. R. & MARTINS DA SILVA, S. J. 2014. Clinically relevant enhancement of human sperm motility using compounds with reported phosphodiesterase inhibitor activity. *Human Reproduction,* 29**,** 2123-2135.

TASH, J. S. 1990. Role of cAMP, calcium, and protein phosphorylation in sperm motility. *Controls of sperm motility: biological and clinical aspects.***,** 229-240.

TERSTAPPEN, G. C., RONCARATI, R., DUNLOP, J. & PERI, R. 2010. Screening technologies for ion channel drug discovery. *Future Medicinal Chemistry,* 2**,** 715-730.

TESARIK, J. 1996. Progesterone-induced acrosome reaction: one receptor is not enough. *Fertility and sterility,* 65**,** 1265-1267.

TESARIK, J., MENDOZA, C. & CARRERAS, A. 1992. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reactions in human sperm. *Fertility and sterility,* 58**,** 1185-1190.

TESARIKI, J., THÉBAULT, A. & TESTART, J. 1992. Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. *Human Reproduction,* 7**,** 1257-1263.

TESAŘÍK, J., OLTRAS, C. M. & TESTART, J. 1990. Effect of the human cumulus oophorus on movement characteristics of human capacitated spermatozoa. *Journal of Reproduction and Fertility,* 88**,** 665-675.

THONNEAU, P., MARCHAND, S., TALLEC, A., FERIAL, M.-L., DUCOT, B., LANSAC, J., LOPES, P., TABASTE, J.-M. & SPIRA, A. 1991. Incidence and main causes of infertility in a resident population (1 850 000) of three French regions (1988–1989)*. *Human Reproduction,* 6**,** 811-816.

TOMLINSON, M., LEWIS, S. & MORROLL, D. 2013. Sperm quality and its relationship to natural and assisted conception: British Fertility Society Guidelines for practice. *Human Fertility,* 16**,** 175-193.

TOMLINSON, M., TURNER, J., POWELL, G. & SAKKAS, D. 2001. One-step disposable chambers for sperm concentration and motility assessment : how do they compare with the World Health Organization ' s recommended methods ? 16**,** 121-124.

TOMLINSON, M. J., KESSOPOULOU, E. & BARRATT, C. L. 1999. The diagnostic and prognostic value of traditional semen parameters. *Journal of Andrology,* 20**,** 588-593.

TOMLINSON, M. J., POOLEY, K., SIMPSON, T., NEWTON, T., HOPKISSON, J., JAYAPRAKASAN, K., JAYAPRAKASAN, R., NAEEM, A. & PRIDMORE, T. 2010. Validation of a novel computer-assisted sperm analysis (CASA) system using multitarget-tracking algorithms. *Fertility and sterility,* 93**,** 1911-20.

TOURNAYE, H. 2012. Male factor infertility and ART. *Asian journal of andrology,* 14**,** 103-8.

TOURNAYE, H. J. & COHLEN, B. J. 2012. Management of male-factor infertility. *Best Practice & Research Clinical Obstetrics & Gynaecology,* 26**,** 769-775.

TSIEN, R. W., ELLINOR, P. T. & HORNE, W. A. 1991. MOLECULAR DIVERSITY OF VOLTAGE-DEPENDENT CA2+ CHANNELS. *Trends in Pharmacological Sciences,* 12**,** 349-&.

UEHARA, Y., MINOWA, O., MORI, C., SHIOTA, K., KUNO, J., NODA, T. & KITAMURA, N. 1995. PLACENTAL DEFECT AND EMBRYONIC LETHALITY IN MICE LACKING HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR. *Nature,* 373**,** 702-705.

UHLER, M. L., LEUNG, A., CHAN, S. Y. & WANG, C. 1992. Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. *Fertility and sterility,* 58**,** 1191-1198.

ULLRICH, A. & SCHLESSINGER, J. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell,* 61**,** 203-212.

VAN VOORHIS, B. J., BARNETT, M., SPARKS, A. E., SYROP, C. H., ROSENTHAL, G. & DAWSON, J. 2001. Effect of the total motile sperm count on the efficacy and cost-effectiveness of intrauterine insemination and in vitro fertilization. *Fertility and sterility,* 75**,** 661-8.

VAN WEERT, J. M., REPPING, S., VAN VOORHIS, B. J., VAN DER VEEN, F., BOSSUYT, P. M. M. & MOL, B. W. J. 2004. Performance of the postwash total motile sperm count as a predictor of pregnancy at the time of intrauterine insemination: a meta-analysis. *Fertility and Sterility,* 82**,** 612-620.

VASTA, V., SONNENBURG, W. K., YAN, C., SODERLING, S. H., SHIMIZU-ALBERGINE, M. & BEAVO, J. A. 2005. Identification of a New Variant of PDE1A Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterase Expressed in Mouse Sperm. *Biology of Reproduction,* 73**,** 598-609.

VISCONTI, P. E. 2009. Understanding the molecular basis of sperm capacitation through kinase design. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 667-8.

VISCONTI, P. E., BAILEY, J. L., MOORE, G. D., PAN, D., OLDS-CLARKE, P. & KOPF, G. S. 1995a. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development (Cambridge, England),* 121**,** 1129-37.

VISCONTI, P. E., JOHNSON, L. R., OYASKI, M., FORNES, M., MOSS, S. B., GERTON, G. L. & KOPF, G. S. 1997. Regulation, localization, and anchoring of protein kinase a subunits during mouse sperm capacitation. *Developmental Biology,* 192**,** 351-363.

VISCONTI, P. E., KRAPF, D., DE LA VEGA-BELTRÁN, J. L., ACEVEDO, J. J. & DARSZON, A. 2011. Ion channels, phosphorylation and mammalian sperm capacitation. *Asian journal of andrology,* 13**,** 395-405.

VISCONTI, P. E., MOORE, G. D., BAILEY, J. L., LECLERC, P., CONNORS, S. A., PAN, D. Y., OLDSCLARKE, P. & KOPF, G. S. 1995b. CAPACITATION OF MOUSE SPERMATOZOA .2. PROTEIN-TYROSINE PHOSPHORYLATION AND CAPACITATION ARE REGULATED BY A CAMP-DEPENDENT PATHWAY. *Development,* 121**,** 1139-1150.

VISCONTI, P. E., MOORE, G. D., BAILEY, J. L., LECLERC, P., CONNORS, S. A., PAN, D. Y., OLDSCLARKE, P. & KOPF, G. S. 1995c. Capacitation of Mouse Spermatozoa. 2. Protein-Tyrosine Phosphorylation and Capacitation are Regulated by a cAMP-dependent Pathway. *Development,* 121**,** 1139-1150.

VISCONTI, P. E., WESTBROOK, V. A., CHERTIHIN, O., DEMARCO, I., SLEIGHT, S. & DIEKMAN, A. B. 2002. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *Journal of reproductive immunology,* 53**,** 133-50.

VISSER, L., WESTERVELD, G. H., XIE, F., VAN DAALEN, S. K. M., VAN DER VEEN, F., LOMBARDI, M. P. & REPPING, S. 2011. A comprehensive gene mutation screen in men with asthenozoospermia. *Fertility and Sterility,* 95**,** 1020-1024.e9.

WALENSKY, L. D. & SNYDER, S. H. 1995. Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. *The Journal of Cell Biology,* 130**,** 857-869.

WALSCHAERTS, M., BUJAN, L., PARINAUD, J., MIEUSSET, R. & THONNEAU, P. 2013. Treatment discontinuation in couples consulting for male infertility after failing to conceive. *Fertility and Sterility*.

WANG, G., GUO, Y., ZHOU, T., SHI, X., YU, J., YANG, Y., WU, Y., WANG, J., LIU, M., CHEN, X., TU, W., ZENG, Y., JIANG, M., LI, S., ZHANG, P., ZHOU, Q., ZHENG, B., YU, C., ZHOU, Z., GUO,

X. & SHA, J. 2013. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *Journal of proteomics,* 79**,** 114-22.

WANG, H., DEY, S. K. & MACCARRONE, M. 2006. Jekyll and Hyde: Two Faces of Cannabinoid Signaling in Male and Female Fertility. *Endocrine Reviews,* 27**,** 427-448.

WEIDINGER, S., MAYERHOFER, A., KUNZ, L., ALBRECHT, M., SBORNIK, M., WUNN, E., HOLLWECK, R., RING, J. & KOHN, F. M. 2005. Tryptase inhibits motility of human spermatozoa mainly by activation of the mitogen-activated protein kinase pathway. *Human Reproduction,* 20**,** 456-461.

WELSBY, P. J., WANG, H., WOLFE, J. T., COLBRAN, R. J., JOHNSON, M. L. & BARRETT, P. Q. 2003. A Mechanism for the Direct Regulation of T-Type Calcium Channels by Ca2+/Calmodulin-Dependent Kinase II. *The Journal of Neuroscience,* 23**,** 10116-10121.

WENNEMUTH, G., CARLSON, A. E., HARPER, A. J. & BABCOCK, D. F. 2003. Bicarbonate actions on flagellar and Ca2+-channel responses: initial events in sperm activation. *Development,* 130**,** 1317-1326.

WHO, W. H. O. 1987. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. Cambridge University Press: New York, New York, USA; Cambridge, England, Uk. Illus. Paper*.

WHO, W. H. O. 1992. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction,* Cambridge, Cambridge University Press.

WHO, W. H. O. 2010a. Induced acrosome reaction assay. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction / Edition 5.* 5 ed.: World Health Organization.

WHO, W. H. O. 2010b. Reference values and semen nomenclature.

WHO, W. H. O. 2010c. Semen Analysis: Preparing the Samples. *WHO Laboratory Manual for Examintation of Human Semen and Sperm-Cervical Mucus Interaction/ Edition 5.* 5 ed.: World Health Organization.

WHO, W. H. O. 2010d. WHO Laboratory Manual for Examination of Human Semen and Sperm-Cervical Mucus Interaction/ Edition 5. 5 ed.: World Health Organization.

WHO, W. H. O. 2010e. *WHO laboratory manual for the Examination and processing of human semen,* Switzerland, World Health Organization.

WHO, W. H. O. 2010f. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction / Edition 5. 5 ed.: World Health Organization.

WILLIAMS, A. C. & FORD, W. C. L. 2001. The Role of Glucose in Supporting Motility and Capacitation in Human Spermatozoa. *Journal of Andrology,* 22**,** 680-695.

WILTSHIRE, E. J., FLAHERTY, S. P. & COUPER, R. T. L. 2000. Hepatocyte growth factor in human semen and its association with semen parameters. *Human Reproduction,* 15**,** 1525-1528.

WONG, C.-H. & YAN CHENG, C. 2005. Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: A review of recent data. *Developmental Biology,* 286**,** 1-15.

XIA, J., REIGADA, D., MITCHELL, C. H. & REN, D. 2007. CATSPER Channel-Mediated Ca2+ Entry into Mouse Sperm Triggers a Tail-to-Head Propagation. *Biology of Reproduction,* 77**,** 551-559.

XIE, F., EDDY, E. M. & CONTI, M. 2013. Chapter Six - Analysis of Signaling Pathways Controlling Flagellar Movements in Mammalian Spermatozoa. *In:* WALLACE, F. M. (ed.) *Methods in Enzymology*. Academic Press.

XIE, F., GARCIA, M. A., CARLSON, A. E., SCHUH, S. M., BABCOCK, D. F., JAISWAL, B. S., GOSSEN, J. A., ESPOSITO, G., VAN DUIN, M. & CONTI, M. 2006. Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. *Developmental Biology,* 296**,** 353-362.

YAN, C., ZHAO, A. Z., SONNENBURG, W. K. & BEAVO, J. A. 2001. Stage and Cell-Specific Expression of Calmodulin-Dependent Phosphodiesterases in Mouse Testis. *Biology of Reproduction,* 64**,** 1746-1754.

YANAGIMACHI, R. 1970. The movement of golden hamster spermatozoa before and after capacitation. *Journal of Reproduction and Fertility,* 23**,** 193-196.

YANAGIMACHI, R. 1994. *Mammalian fertilisation,* New York, Raven Press.

YAO, Y. Q., NG, V., YEUNG, W. S. B. & HO, P. C. 1996. Profiles of sperm morphology and motility after discontinuous multiple-step Percoll density gradient centrifugation. *Andrologia,* 28**,** 127-131.

YOVICH, J., EDIRISINGHE, W., CUMMINS, J. & YOVICH, J. 1990. Influence of pentoxifylline in severe male factor infertility. *Fertility and Sterility,* 53**,** 715.

YOVICH, J. L. 1993. PENTOXIFYLLINE - ACTIONS AND APPLICATIONS IN ASSISTED REPRODUCTION. *Human Reproduction,* 8**,** 1786-1791.

YUASA, K., KANOH, Y., OKUMURA, K. & OMORI, K. 2001. Genomic organization of the human phosphodiesterase PDE11A gene. *European Journal of Biochemistry,* 268**,** 168-178.

ZHOU, C.-X., WANG, X.-F. & CHAN, H.-C. 2005. Bicarbonate secretion by the female reproductive tract and its impact on sperm fertilizing capacity. *Shengli Xuebao,* 57**,** 115-124.

ZHU, M. X., MA, J., PARRINGTON, J., CALCRAFT, P. J., GALIONE, A. & EVANS, A. M. 2010a. Calcium signaling via two-pore channels: local or global, that is the question. *American Journal of Physiology - Cell Physiology,* 298**,** C430-C441.

ZHU, M. X., MA, J., PARRINGTON, J., GALIONE, A. & MARK EVANS, A. 2010b. TPCs: Endolysosomal channels for Ca2+ mobilization from acidic organelles triggered by NAADP. *FEBS Letters,* 584**,** 1966-1974.